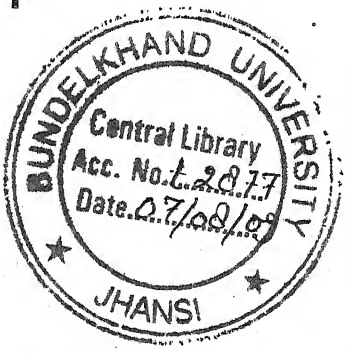
COMPARATIVE PHYSICO-KINETICS OF β-GLUCOSIDASES FROM SELECTED MEDICINAL PLANTS

THESIS

SUBMITTED TO THE
BUNDELKHAND UNIVERSITY



FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

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2008

Certificate

This is to certify that this dissertation work entitled "**Comparative Physico-Kinetics of β-Glucosidases from Selected Medicinal Plants**", submitted by Siddhartha Kumar Mishra, M. Sc. (Biochemistry) for the award of Ph.D. degree in Biochemistry, embodies the bonafide work carried out by him under our joint supervision. He fulfills all the laid down requirements regarding the nature and prescribed period of work of the Bundelkhand University, Jhansi, for the degree of Doctor of Philosophy in Biochemistry. The data presented in this thesis are all original, unless stated otherwise and have been checked from time to time by both of us and we are satisfied about the genuineness.

We recommend this thesis for submission to award him Ph.D. degree.

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LUCKNOW.

17/04/2008

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Dedicated

to my

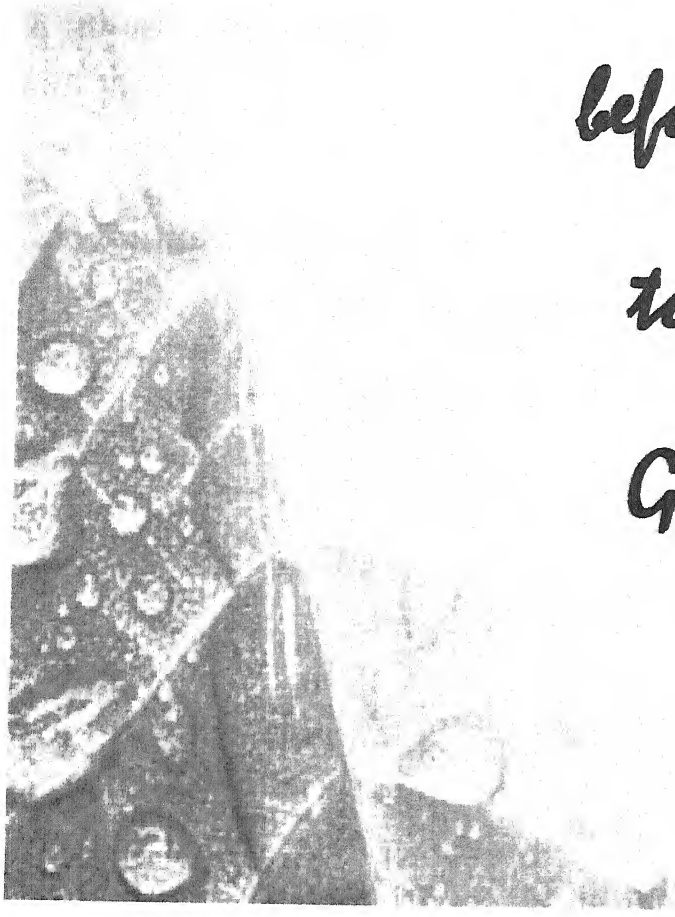
Parents

and

before them

to my

Grandparents



Acknowledgement

I would like to express my sincere gratitude and profound respect to Dr. R. S. Sangwan, Scientist-F (Deputy Director), Plant Physiology & Biochemistry Division, Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow (U.P.) India and Dr. M. K. Srivastava, Reader and Head, Department of Biochemistry, J. C. Bose Institute of Life Sciences, Bundelkhand University, Jhansi (U.P.) India, under whose joint supervision this research work for the thesis was carried out.

I might suffer from words thanking Dr. Sangwan for igniting the research of protein biochemistry in me and I shall always remain indebted to him for his able guidance throughout my study. He served as an excellent mentor in every aspect of my research work via scientific motivation, and morale support, his constant interest, and care. "Thank you Sir for having confidence in me".

I can't express enough gratitude to Dr. Srivastava who helped me to accomplish my research work by providing his valuable advice and allowing me to gain more knowledge. I am truly grateful for his kind support, encouragement, and constant guidance.

Sometimes I felt like a researcher, at times a learner, and a kid, and of course all this was due to Dr. (Mrs.) N. S. Sangwan, I feel truly fortunate to have had such an outstanding scientist and a caring person in laboratory. I shall always remain thankful to her for her valuable comments, tips and suggestions along with criticism.

I would also like to extend a sincere thank to Dr. S. P. S. Khanuja, Director, CIMAP, for his keen support and provision of research facilities at the institute.

I would like to thank Dr. A. H. A. Farooqi, Head, Plant Physiology & Biochemistry Division, CIMAP, for his helpful nature and valuable suggestions throughout my research program.

I'm especially thankful to the Council of Scientific and Industrial Research (CSIR), New Delhi for providing me the award of Senior Research Fellowship (SRF) which helped me to shape-up my research career.

Mr. Avdhesh Kumar Srivastava is genuinely thanked for his constant support in maintaining plants and laboratory. I also thank the scientists and staff of other departments of CIMAP for their timely help in my research work.

I cordially thank Prof. U. N. Dwivedi, Prof. P. C. Misra, Prof. M. K. Misra, Dr. R. K. Mishra, Dr. Sameer Sharma and other teachers of Department of Biochemistry, University of Lucknow for guiding me throughout my research career.

I wish to thank all my colleagues and friends who came across my research work and helped me in direct or indirect ways. Pankaj is thanked for his scientific and literary discussions and a good

company, and Farzana & Sumbul for making the laboratory milieu ever cheering-flickering. Rajiv, Manoj, Mahesh, Priyanka, Shilpa, Sanjeev, Kavita, Kirti, Deepti, Abhishek, Rajendra, Anil, Om Prakash, Sandeep, and Jyoti are thanked for their requisite assistance and support.

I also thank Digvijay Singh, Subhash Gupta, Arvind Tripathi, Sanjay Thul, Ankur Garg, Rakesh Upadhyay, Yougal Kishore, Mukesh, Vijay Raju, Ratnesh, Shrawan, Richa Pandey and Archana Pandey for their friendly support.

I owe very special thanks to Dr. Vishal Trivedi for helping me in a "friend-philosopher and guide" mode and to his family.

My friends Praveen and Gaurav are specially thanked for accompanying me to movies almost every weekend. In friends row I also thank to Rahul, Utkarsh, Mayank, Vikas, Manish and other friends not listed herein.

The acknowledgement would not be complete without mentioning the warmth and support of my relatives, and my Family specially my brothers and sisters (Shambhavi and Kartikeya; Animesh, Aaditya, Anya and Aashutosh) for their rejuvenating corporation at home.

"And above all, I soulfully thank GOD for strengthening me and my faith in Him."

Date: June 17th *2008*
Place: CIMAP, Lucknow

Siddhartha .

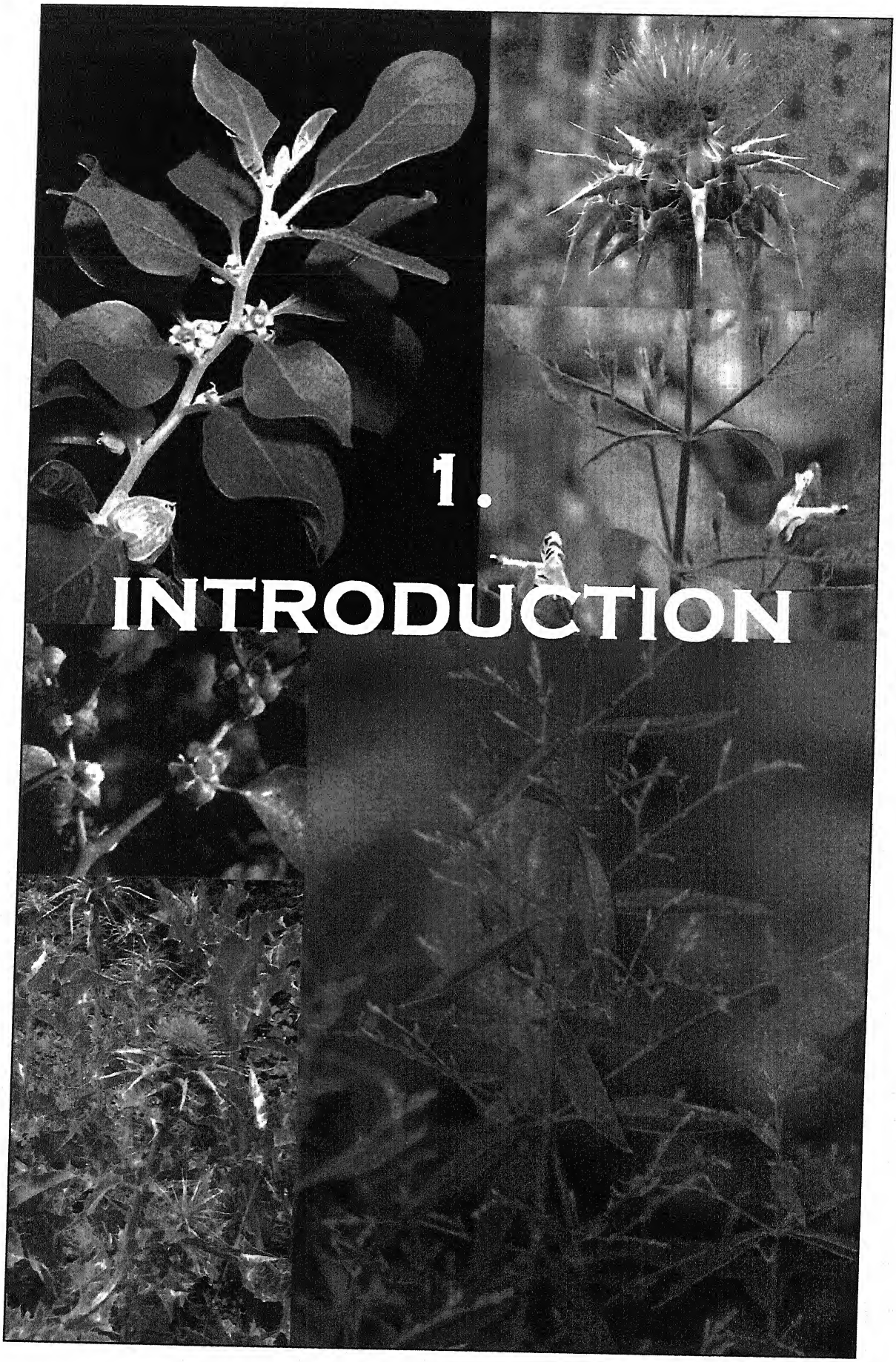
(Siddhartha Kumar Mishra)

ABBREVIATIONS

A_{280}	absorbance at 280 nm
BSA	bovine serum albumin
cal	calories
cm	centimetre
DCPIP	2,6-dichlorophenol indophenol
d	day
E_a	energy of activation
EC	enzyme commission
EDTA	ethylenediaminetetra aceticacid
EGTA	ethyleneglycoltetra aceticacid
f.wt.t.	fresh weight tissue
g	gram
h	hour
IU	international unit
K_{av}	average partition coefficient
kDa	kilodalton
Kg	kilogram
K_m	Michaelis constant
K_{cat}	turn over number
L	litre
m	metre
M	molar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
M_r	molecular weight
nm	nanometer
°C	degree celsius
PAGE	polyacrylamide gel electrophoresis
PCMB	*p*-chloromercuric benzoic acid
PMSF	phenylmethyl sulfonyl fluoride
PVPP	polyvinylpolypyrolidone
SDS	sodium dodecyl sulfate
T	absolute temperature
TEMED	N,N,N′,N′-tetramethyl ethylene diamine

Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
v	volt
v/v	volume by volume
w	watt
w/v	weight by volume
β-ME	beta-mercaptoethanol
μg	microgram
μl	microlitre
ε°	extinction coefficient

CONTENTS



1.
INTRODUCTION

β-Glucosidase (β-D-glucoside glucohydrolases; EC 3.2.1.21) catalyzes hydrolysis of β-glucosidic linkages of glucosides. The hydrolytic action on of glycosidic linkages between the hemiacetal-OH group of a cyclic aldose or glucose and the –OH group of another compound *viz.*, sugar, amino-alcohol, aryl-alcohol or primary, secondary, or tertiary alcohols proceeds *via* the following steps:

1. During glycosylation, an enzymic nucleophile attacks the anomeric (C_1) center of the substrate glycoside, resulting in formation of a covalently linked α-glycosyl enzyme intermediate through an oxocarbenium ion-like transition state (Withers and Street, 1988). Thus, the anomeric configuration at C_1 is reversed.
2. Another active residue of the enzyme serves as the acid base catalyst and donates a H^+ to the glycosidic oxygen, thereby assisting in departure of the agylcone (or other glycone, as in disaccharides) group.
3. The glycosyl-enzyme intermediate is hydrolyzed *via* general base-catalyzed attack by water at the anomeric center to release β-glucose as the product. The *trans*-addition of an –OH group results in the net retention of the β-anomeric configuration. The nucleophilic residue also acts as the leaving group in the deglycosylation step. Both formation and hydrolysis of the enzyme-glycosyl intermediate occurs *via* an oxocarbenium ion-like transition state.

These glycohydrolases have gained more upstream attention of research recently because of their varied and vital roles in a number of biological processes and attendant potential biotechnological applications. Plant β-glucosidases have been implicated in defense against pests (Conn, 1981; Niemeyer, 1988; Poulton, 1990), phytohormone activation (Wiese and Grambow, 1986; Brzobohaty *et al.*, 1993), lignification (Dharmawardhana *et al.*, 1995), and cell wall catabolism (Leah *et al.*, 1995; Simos *et al.*, 1994). Also, interest in these enzymes has gained momentum owing to their potential biosynthetic abilities. The enzymes exhibit utility in synthesis of diverse oligosaccharides, glycoconjugates, alkyl- and aminoglucosides owing to catalytic action of

transglycosylation under certain conditions. These enzymes are ubiquitously present in living organisms from microbes to plants and animals. However, their physico-kinetics could be immensely varied owing to differential functional recruitment in different organisms.

While physiological functions, characteristics, and catalytic mechanism of action of β-glucosidases are increasing addressed through site-specific mutagenesis and catalytic and structural information on mutated catalytic protein, it needs to be complemented with the naturally existing catalytic variability in the enzyme forms and their structural overtines. The later stems from isolation, purification and characterization of β-gluciosdases from diverse sources, tissue forms and in diverse metabolic backgrounds existing or induced in the organisms. A large number of these enzymes from bacteria, fungi and plants have been purified and studied, and a few of them have been cloned for structural studies.

The computational analysis of protein sequences of β-glucosidase classified it into families 1 and 3 of the glycoside hydrolases. Family 1 of glycosylhydrolases includes β-glucosidases from archaebacteria, plants, and mammals. Family 3 comprises β-glucosidases of some bacterial, mold, and yeast origin. Out of the 82 families classified under glycosylhydrolase category, these belong to family 1 and family 3 and catalyze the selective cleavage of glucosidic bonds (Henrissat, 1991; Henrissat and Bairoch, 1993). β-Glucosidases classification schemes are based on similarity at the structural and molecular levels and the elucidation of their structure-function relationships, directed evolution of existing enzymes toward enhanced thermostability, substrate range, biosynthetic properties, and applications are some of the vast domains of biological studies on β-glucosidases.

In addition to the biotechnological importance of this group of enzymes, study of their catalytic mechanisms provides a means of understanding the catalytic power of the enzyme. Many mechanistic studies have been performed

on β-glucosidase (Legler *et al.*, 1980; Kempton and Withers, 1992, Bauer and Kelly, 1998). However, most of such studies and advances pertain to family 1 enzymes. In summary, the members of this group are retaining enzymes, which catalyze the hydrolysis of their substrates with retention of the anomeric configuration. Although the action of family 3 enzymes is thought to be similar to that of family 1 enzymes, detailed kinetic investigations are not as wide and detail as for the family 1 counterparts.

Plant metabolism is much more complex than the animal and microbial counterparts. Therefore, plant enzyme catalytic properties are implied to have evolved in accordance with the metabolic background of the plant species, often in an individual-specific manner. This may be interrelated to different physiological functions of β-Glucosidases in different plant species, depending on the nature of the aglycone(s) that need to be catalytically processed for deconjugation. Some of the metabolites that could be/are facile to deglycosidation are glycosides and in the overall vanilla aroma, several minor compounds are known to exist in extract of green bean of vanilla (Dignum *et al.*, 2004).

Several of the plant primary and secondary metabolites occur as glucosides e.g. some of the indole alkaloids (e.g. strictosidine), several of flavanoids, plant hormones, hydroxamic acids, flavanols, thiocyanates and mandelonitrils (Niemeyer, 1988). In fact, plant hormone concentration *in vivo* is regulated not only by *de novo* synthesis but also by activation and inactivation mechanisms. One of the mode of inactivation is by glucosylation, and with the exception of ethylene, all plant hormones have been identified as glucosyl conjugates. β-Glucosidases may have different substrate specificities, which presumably relate to their physiological function and thus, enzymes involved may hydrolyze cyanogenic glucosides, cellobiose or gentiobiose. β-Glucosidases may have different functions depending on the aglycone moieties of their physiological substrates. Thus, they exhibit high specificity towards naturally-occurring substrates. Among the oldest work, the enzymatic hydrolysis of the

cyanogenic glycoside amygdalin by an enzyme mixture from almond known as emulsion involved two glycosidases, amygdalin-hydrolase (AH) and prunasin-hydrolase (PH), which catalyze the reaction stepwise by removing two glucose residues from amygdalin. The natural substrates of β-glucosidases include steroid β-glucosides and β-glucosyl ceramides of mammals, cyanogenic β-glucosides and glucosinolates of plant secondary metabolism, and oligosaccharide products released from digestion of the cellulose of plant cell-walls (Clarke *et al.*, 1993). Artificial substrates such as benzyl, nitrophenyl and methylumbelliferyl-glycosides are frequently used during the purification and characterization of glycosidases. These substrates allow investigators to easily assay enzyme velocities of hydrolysis and substrate affinities. However, qualitative and quantitative enzymatic assays on the natural substrates are very few when compared to artificial substrates. Dalcochinase, a β-glucosidase from the seeds of Thai rosewood (*Dalbergia cochinchinensis* Pierre) showed its novel isoflavonoid specificity and the enzyme shows evolutionary relationship to other β-glucosidases. The enzyme not only catalyzes reverse hydrolysis using glucose as substrate, yielding di- and tri-saccharides, but can also transfer glucose from *p*-nitrophenyl-β-D-glucopyranoside to alkyl alcohols proving its transglucosylation activity (Srisomsap *et al.*, 1996).

β-Glucosidase performs very important role in enological applications (wine making). A comparison has been made of the β-glucosidase of a commercial preparation from *Aspergillus niger* chosen for its high activity and that of 463 yeast strains, isolated and screened from Sicilian musts and wines. A higher percentage of β-GS-positive strains (30%) were found on Yeast Carbon Base + lysine compared to 18% on Sabouraud + ethanol + SO_2 and just 8% on Sabouraud medium. The positive strains with the highest β-GS activity were subsequently identified and compared with the purified commercial preparation. Optimum pH values were found to be 4.5 for *A. niger* β-GS that is close to that of wine, whilst amongst the yeasts, AL 41 was the strain with optimum pH closest

to that of wine. Optimum temperatures were found to be about 62°C and 20–25 °C for *A. niger* and yeast β-GS, respectively. The different natural sources of β-glucosidase that have been examined for oligosaccharide synthesis include almond, sesame, cycad, *Fusarium oxysporum, Trichoderma viridae* and *Thermus thermophilus* (Yasukochi *et al.*, 1999; Kannan *et al.*, 2004). The most commonly used donors in these studies were *o/p*-nitrophenyl β-glucoside, cellobiose, laminaribiose and gentiobiose. Specifically mutated glycosidases, also called glycosynthases, have been reported to synthesize oligosaccharides in very high yield. The Reverse to the natural hydrolytic reaction catalyzed by glycosidases, under suitable conditions they can also catalyze transglycosylation reactions.

β-Glucosidases are generally implicated in governing the release of free aglycones which function as intermediates of metabolic pathways or as catabolic products and are volatilized as environmental/ecological signals. These enzymes also participate in a number of primary physiological activities exclusively including flavor and fragrance development and citrus juice-bitterness (due to the β-glucosidase-catalyzed generation of limonoids such as limonin from their glycol-conjugates during crushing). The enzyme is also important in volatile oil plants as a number of terpenols exist as glycoconjugates as is the case for grapes and berries.

Enzymatic hydrolysis of glycosidic bonds is carried out with one of two stereochemical outcomes; net retention or net inversion of anomeric configuration, thus glycosidases are classified as either retaining or inverting. Basic mechanisms for these processes were proposed by Koshland (1953), and these have been elaborated upon in the interim both mechanistically, and particularly structurally. The inverting glycosidases use a direct displacement mechanism through an oxocarbenium ion-like transition state. Catalysis by retaining glycosidases and transglycosylases proceeds *via* a double-displacement mechanism in which a covalent glycosyl–enzyme intermediate is formed and hydrolyzed via oxocarbenium ion-like transition states.

Reports indicate that glucosyl, galactosyl, mannosyl and xylosyl acceptors have been employed for transglucosylation reaction and the acceptors which have been used so far in transglucosylation reactions are limited to free sugars and O- or S-glycosides. Very few reports are available on the synthesis of oligosaccharides catalyzed by almond β-glucosidase and only glucose and galactose acceptors have been employed. Unlike β-galactosidase from *Bacillus circulans*, acceptor specificity of this enzyme remains to be explored. Similarly, efficacy of organic co-solvent in the reaction medium to improve the disaccharide yield has also not been documented so far (Bhatia *et al.*, 2002).

Withania somnifera (Ashwagandha) (Solanaceae) is used since the times of Ayurveda, the ancient system of Indian medicine. From phytochemical point of view, the herb contains a group of biologically active constituents known as withanolides. The chemistry of withanolides has been studied and they are basically steroidal lactones (highly oxygenated C-28 steroid derivatives). They are present in roots and leaves of *Withania somnifera.* Withaferin-A is therapeutically active withanolide reported to be present in leaves. In animal studies, withaferin-A has shown significant anticancer activity. Withanolides are similar to ginsenosides (the active constituents of *Panax ginseng*) in structure and activity and they are believed to be immunomodulator. *Withania somnifera* possesses many other pharmacological important, including anti-inflammatory, antitumor, and immunomodulatory properties, as well as exerting an influence on the endocrine, nervous, and cardiopulmonary systems (Gupta and Rana, 2007). *Withania somnifera* may be useful in many ailments, including arthritis and other musculo-skeletal disorders, stress-induced nervous exhaustion, and hypertension. There are a few preliminary studies available on the effects of *Withania* on the immune system, central nervous system, hemopoetic system, and general growth promotion to form a basis for further studies on metabolites of *Withania* and to find out its functionality in animal models especially humans. The use of *Withania somnifera* as a general tonic to increase energy and prevent

disease may be partially related to its effect on the immune system. Some of the withanolides exist as glycosylated entities. Glycowithanolides including sitoindosides IX and X have been shown to possess immunomodulatory and neurological (antistress, memory, and learning) activities in Swiss mice and Wistar strain albino rats (Prakash *et al.*, 2002).

Andrographis paniculata (Kalmegh) (Acanthaceae) is a traditional medicine used for the treatment of cold, fever, laryngitis and infections diseases ranging from malaria to dysentery diarrhea in China, India and other Southeastern Asian countries. In addition, it is claimed to possess immunological, antibacterial, anti-inflammatory, antivenin, antithrombotic and hepatoprotective properties. The plant contains a characteristic group of secondary metabolites of diterpenoid category called andrographolide. The extract of *Andrographis* and the major bioactive constituents contributes to a broad range of effects including antibacterial, anti-inflammatory and immunostimulant effects and was reported to inhibit lipid peroxidation. It is a prominent component of many traditional Indian and Chinese formulae for treatment of liver disorders, protective effects against damage of the liver produced in mice by giving them carbon tetrachloride, alcohol or other chemicals (Kapil *et al.*, 1993). The contents of andrographolide, neoandrographolide and dehydroandrographolide are the criterion for estimating the quality of *A. paniculata*. The primary medicinal component andrographolide has a very bitter taste, is a colorless crystalline in appearance, and is a "diterpene lactone" in nature-a chemical name that describes its ring like structure (Matsuda *et al.*, 1994). Both growing region and seasonal variability play an important role in formation of these metabolites in plants so as to the concentration of these diterpene lactones. Andrographolide is found in the whole plant but is most concentrated in the leaves. It is a diterpene containing a γ-lactone ring connected to a decalin ring system via an unsaturated C-2 moiety. It has multiple pharmacological activities such as protozoacidal, antihepatotoxic, anti-HIV, anticancer, antitumor, hypoglycemic and hypotensive activities.

Andrographolide has showed good activity *in vivo* breast cancer models, whose anticancer activity is thought to be exerted through blockage of cell cycle progression by the induction of cyclin-dependent kinase inhibitors (CDK1) and with a concomitant decrease in cyclin-dependent kinase (CDK4) expression. Recent studies suggested that andrographolide is an interesting pharmacophore with anticancer and immunomodulatory activities and hence has the potential to be developed as an anticancer chemotherapeutic agent (Calabrese *et al.*, 2000).

Silybum marianum (L.) Gaertn. is a member of the aster family (Asteraceae), which is commonly known as "milk thistle" due to its characteristic flower shape. Silymarin is major flavonoid molecule derived from the plant, has been used for centuries as a natural remedy for diseases of the liver and biliary tract. Silymarin and its active constituent, silybin, have been reported to work as antioxidants scavenging free radicals and inhibiting lipid peroxidation. Studies also suggest that they protect against genomic injury, increase hepatocyte protein synthesis, decrease the activity of tumor promoters, stabilize mast cells, chelate iron, and slow calcium metabolism. Silymarin has been traditionally used in the treatment of liver disease and is regularly used for an assortment of liver diseases including cirrhosis and viral hepatitis in humans (Kren *et al.*, 1998). The only actual studies conducted in dogs have concerned mushroom poisoning as above and other uses in pet species are inferred from human use. Milk thistle extracts appear to be safe to use; however, their benefit is not well defined scientifically (Kren and Walterova, 2005). *Silybum* has been used since long treating/recovering after; chronic liver disease, liver rehabilitation after hepatitis, gall-bladder disease, alcohol, drug, dietary abuse, exposure to chemicals that threaten normal liver function, poor milk production in feeding mothers, jaundice, varicose veins, and allergies associated with liver damage. The silybin component of sillymarin has been related to cholesterol-lowering effects. Through the capability to increase bile solubility, sylimarin may also help prevent or alleviate gallstones. Both silymarin and silybin have been shown to exhibit preventive effects against

photo-carcinogenesis in various animal tumor models. Topical application of silymarin to mouse skin (SKH-1 hairless mouse model) reduced UVB induced tumor incidence, tumor multiplicity and tumor size compared to those of non-treated animals. Silybin inhibited photocarcinogenesis in mice when applied topically or administered in the diet (Mallikarjuna *et al.*, 2004).

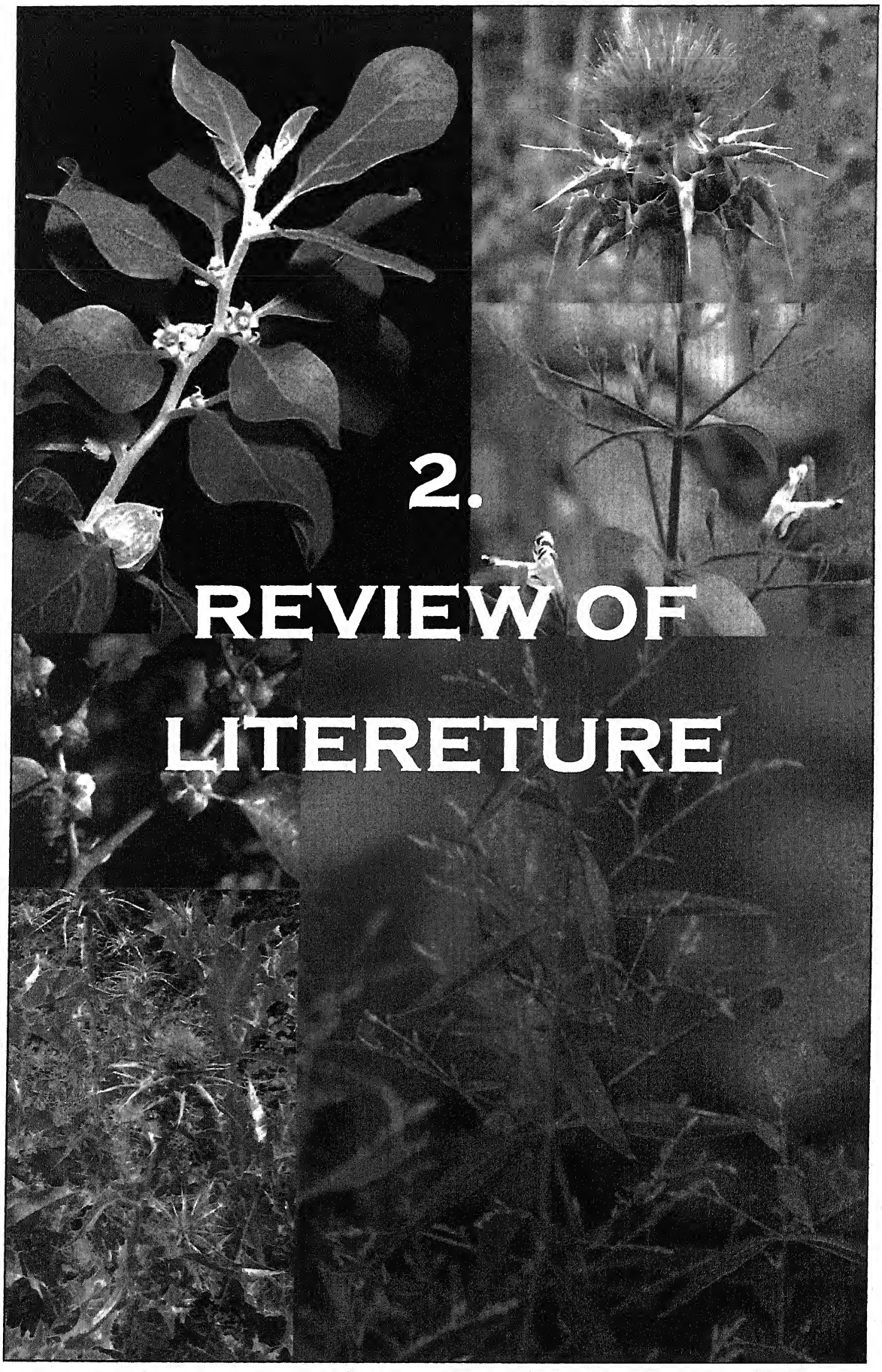
In summation, the specific secondary metabolites of these plants exist in glucosylated form invoking a role of glucosidase in controlling the release of the molecule in native form for physiological functions. Thus, abundance and characteristics of these enzymes are important parameters to governing the inter-conversions. Therefore in this study, it is proposed to study physico-kinetic characteristics of β-glucosidases in these selected medicinal plants.

Accordingly, it was planned to carryout the isolation, purification and physico-kinetic characterization of a β-glucosidase from the *Withania somnifera, Andrographis paniculata* and *Silybum marianum* to comprehend a comparative account. The technical program of work for the research work consisted of:

- profiling of β-glucosidase level in different plant parts in the selected medicinal plants, isolation of β-glucosidases from selected medicinal plants, purification of β-glucosidases from selected medicinal plants,
- elucidation of comparative physico–kinetic characteristics of catalysis by the selected enzymes, substrate specificity and regulatory properties of the purified enzymes, molecular characterization of purified enzyme with respect to limited amino acid sequence or peptide mapping, exploration of catalytic feasibility to carry out transglycosylation under designed conditions, if functional.
- Purification and physico-kinetic characterization of the β-glucosidases from selected medicinal plants and discerning subunit molecular weight.

- Comparative proteomic analysis of the β-glucosidases with reference to their application in plant system. Application of the β-glucosidases in different metabolic conditions with reference to substrate specificity.

The phytochemicals from selected medicinal plants have diverse application in secondary metabolism and especially the aglycone generation through glucosidic cleavage by glucosidase. This secondary metabolomics has emphasizes to study the β-glucosidase of the plant and to find out their functional physico-kinetic characteristics. This is in consequence to raise a library of novel plant β-glucosidase for overall enzymatic analyses.



2. REVIEW OF LITERETURE

The overall biological system depends on a complex network of chemical reactions catalyzed by specific enzymes, and any modification of the enzyme pattern may have drastic consequences for a living organism. The mechanisms by which enzymes catalyze chemical reactions are in itself one of the most fascinating fields of scientific investigation being pursued at the present time. Thus, enzymes, as catalysts, have received increasing attention from many scientific fields, especially biochemistry, biotechnology and molecular biology, microbiology, genetics, botany and agriculture, pharmacology and toxicology, medicine, and chemical engineering. Due to the complex structure and biological and physical instability of these macromolecules the mechanics of enzyme catalysis is still quite limited. Biochemical studies on enzymes provide the source for understanding how enzymes function and how the catalytic sites react with substrates. Enzymes are key components in every metabolic pathway that occurs within the cell. Thus, investigating enzymes provides valuable information about the catalytic mechanism and function in metabolism. Catalysis is intimately related to the molecular interactions that take place between a substrate and a specific part of the enzyme molecule. Today, spectroscopic methods, x-ray crystallography, and more recently, multidimensional NMR methods, provide a wealth of structural insights on which theories of enzyme mechanisms can be built.

The ability of β-glucosidases to activate glycosidic bonds renders the enzyme highly interesting as a promising biocatalyst for the metabolism of stereo/regio-specific glycosides or oligosaccharides, molecules potentially useful as functional materials, nutraceuticals, or pharmaceuticals because of their biosignaling, recognition, or antibiotic properties. Opposite to its hydrolytic activity, it may catalyze a glycosidic bond formation *via* either a thermodynamically controlled "reverse" hydrolysis or a kinetically controlled transglycosylation. The main advantage of using β-glucosidases over glycosyltransferase in such applications is that the former does not require any

involvement of expensive/unstable nucleotide sugars precursors as well as its wide functionality even under harsh reaction conditions (Bhatia *et al.*, 2002).

Glycosides are compounds containing a carbohydrate and a noncarbohydrate residue in the same molecule. The carbohydrate residue is attached by an acetal linkage at carbon atom 1 to a noncarbohydrate residue or aglycone. The carbohydrate component is called the glycone. If the carbohydrate portion is glucose, the resulting compound is a glucoside. Many of these compounds are pharmacological important molecules or possess other appealing properties.

The aglycone may be methyl alcohol, glycerol, a sterol, a phenol, etc. An acetal has two ether functions at a single carbon atom.

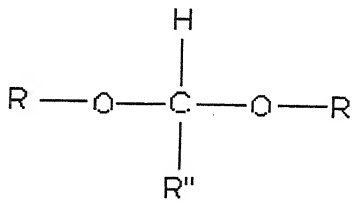


Figure 1: A simple aglycone skeleton

The glycone can be attached to the aglycon in many different ways. The most common bridging atom is oxygen (*O*-glycoside), but it can also be sulphur (*S*-glycoside), nitrogen (*N*-glycoside) or carbon (*C*-glycoside). In general, one distinguishes between α-Glycosides and β-glycosides, depending on the configuration of the hemiactal hydroxyl group. The majority of the naturally occurring glycosides are β-glycosides. Generally glycosides are more polar than the aglycones and as a result glycoside formation usually increases water solubility. This may allow the producing organism to transport and store the glycoside more efficiently.

2.1. Classification and distribution of glycosides

Many biologically active compounds are glycosides. The pharmacological effects are largely determined by the structure of the aglycone. Glycosides comprise several important classes of compounds such as hormones, sweeteners, alkaloids, flavonoids and antibiotics (Kren and Martankova, 2001).

Depending up on the chemical nature of the aglycone the glycosides are classified in Figure 2.

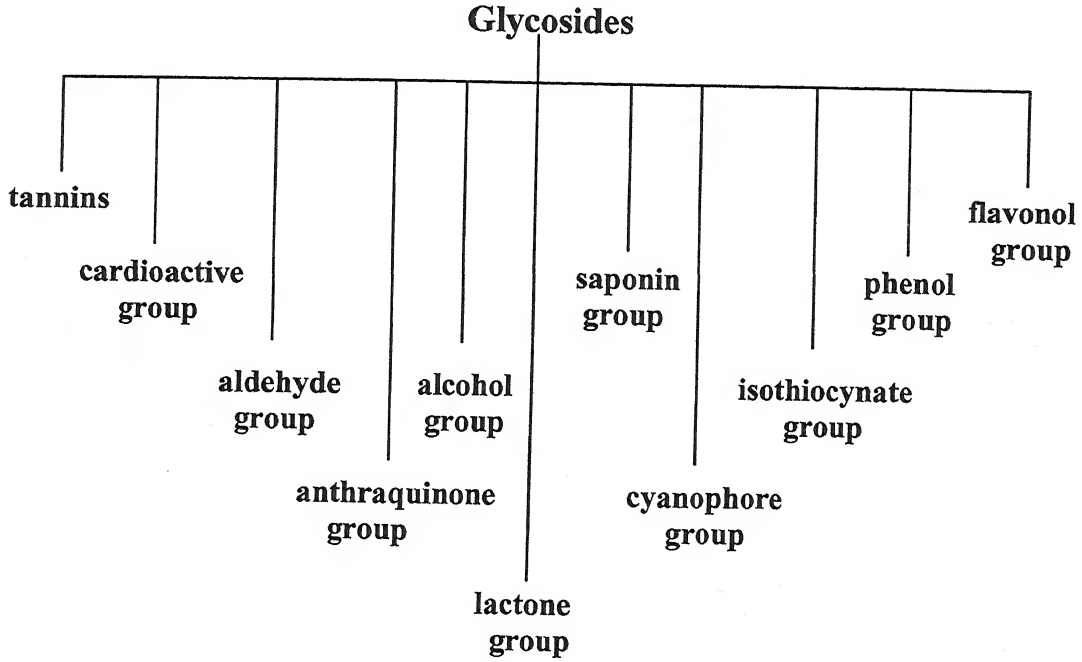


Figure 2: Group-wise classification of glycosides

2.1.1. Saponin glycosides

Saponin glycosides are divided into two types based on the chemical structure of their aglycones (sapogenins). Saponins on hydrolysis yield an aglycone known as "sapogenin".

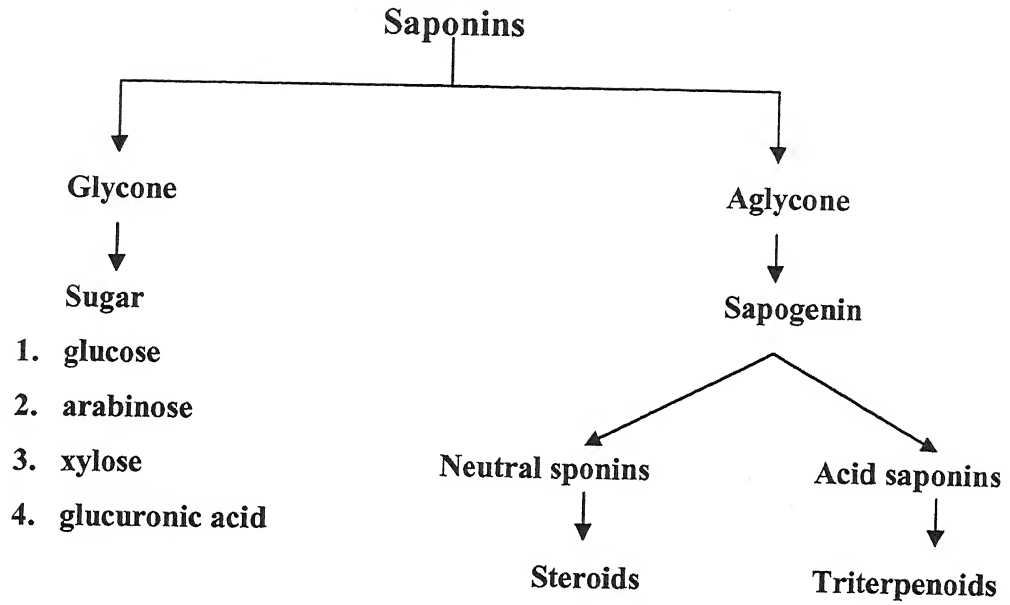


Figure 3: Distribution of saponins.

The main pathway is schematically presented in Figure 4; leading to biosynthesis of both types of sapogenins is similar and involves the head-to-tail coupling of acetate units. However, a branch occurs, after the formation of the triterpenoid hydrocarbon, squalene, that leads to steroids in one direction and to cyclic triterpenoids in the other.

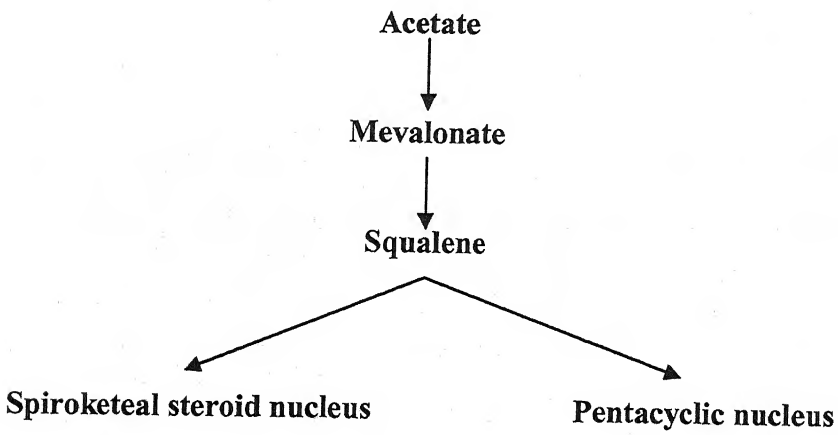


Figure 4: Outline of mevalonate pathway.

2.1.2. Cyanogenic glycosides

The cyanogenic glycosides belong to the products of secondary metabolism, to the natural products of plants. These compounds are composed of an α-hydroxynitrile type aglycone and of a sugar moiety (mostly D-glucose). The distribution of the cyanogenic glycosides (CGs) in the plant kingdom is relatively wide, the number of CG-containing taxa is at least 2500, and a lot of such taxa belong to families Fabaceae, Rosaceae, Linaceae, Compositae and others. The biosynthetic precursors of the CGs are different L-amino acids, these are hydroxylated then the N-hydroxylamino acids are converted to aldoximes, these are turned into nitriles. The last ones are hydroxylated to a-hydroxynitriles and then they are glycosilated to CGs. The generation of HCN from CGs is a two step process involving a deglycosilation and a cleavage of the molecule (regulated by β-glucosidase and α-hydroxynitrilase). Vetter (2000) also reported that some plant species like cassava (*Manihot esculenta*) have the ability to produce cyanides and are strong cytotoxins, competitive inhibitors of the Fe^{3+} of the heme group. The cells detoxify them by glycosylation, i.e. by linking them β-glycosidically to sugar residues (usually glucose).

Most plants produce a small amount of cyanide associated with ethylene production between 3000-12000 plant species produce suffcient quantities of cyanogenic compounds (McMahon *et al.*, 1995). The enzymic hydrolysis produces the aglycone and the sugar moiety. The CGs can be grouped according to chemical nature of substituents, namely aliphatic, aromatic groups and into the glycosides with a free α-hydroxynitrile. Some of these CGs are better known than the others because the carrying plant species (group) has a greater practical importance, several economically important plants are highly cyanogenetic (linamarin in *Manihot esculenta, Linum usitatissimum, Trifolium repens,* dhurrin in *Sorghum* species, amgydalin in rosaceous plants, lotaustralin in *Lotus corniculatus,* etc.). The linamarin and lotaustralin have a relatively broad distribution in the plant kingdom; have been demonstrated by Seigler *et al.* (1989) in the following

plant families: Compositae, Euphorbiaceae, Linaceae, Papaveraceae and Fabaceae (Leguminosae). Conversely, it is generally true with few exceptions that only one or two characteristic glycoside will occur in a given plant family (Poaceae: dhurrin; Compositae: linamarin; Polypodiaceae: prunasin and vicianin, Rosaceae: amygdalin and prunasin). The cyanogenic compounds of plants belong undoubtedly to secondary plant metabolites which have or can have a chemotaxonomical character. The families Saxifragaceae, Rosaceae, Mimosaceae, Fabaceae, Myrtaceae, Linaceae and Euphorbiaceae are in the subclass Rosiidae, other cyanogenous families are in subclasses Ranunculidae (family Papaveraceae), in Lamiidae (families Caprifoliaceae, Sambucaceae, Oleaceae), Asteridae (family Compositae). CGs has been reported by Seigler *et al.* (1989) from many members of the three subfamilies of Fabaceae.

2.2. Synthesis of *C*-aryl glycosides

The *C*-aryl glycosides comprise a subclass of a broader family of naturally occurring *C*-glycosides that have biological activities and are resistant to enzymatic hydrolysis. Some representative members of this class of natural products that are of significant interest include galtamycinone, vineomycinone B2, and kidamycin. The *C*-aryl glycosides may be classified into four different subgroups based upon the orientation of the sugar residue(s) relative to the phenolic hydroxyl group on the aromatic ring (Martin, 2003).

2.3.1. Chemical synthesis of glycosides

The reactivity of glycosyl donors and acceptors is highly dependent on their anomeric activation and on the protecting groups that the sugar ring carries (Davis, 2000). Recently, Ye and Wong (2000) reported the methods to quantify the relative reactivities of glycosyl donors on the basis of experimentally obtained reaction rates to the synthesis of a small (33 member) library of trisaccharides and tetrasaccharides. A very important issue in carbohydrate synthesis is the

formation of the desired α- or β-anomeric linkage. Some attempts have been made to direct the formation of otherwise unfavourable linkages through tethering of glycosyl donor and acceptor before formation of the glycosidic linkage. There is a great interest in the polymer-supported synthesis of oligosaccharides and glycoconjugates with potential applications in library and automated synthesis (Ye and Wong, 2000).

2.3.2. Enzymatic synthesis of glycosides

Enzymatic synthesis of glycosides is to be considered of great interest with respect to β-glucosidase, which is meant to break such natural glycosides. The presented work comprises the enzymatic functionality exploration and thus it becomes highly important to emphasise the enzymatic synthesis glycosides.

There are two classes of biocatalysts available for the enzymatic synthesis of glycosides: the glycosidases or transglycosidases and the glycosyltransferases. Glycosidases have the advantage that they are often readily available and use simple glycosyl donors. Because glycosidases usually catalyse the reverse reaction (hydrolysis), reaction conditions need to be carefully chosen to encourage synthesis, for example by using a large excess of acceptor and an activated donor. Glycosidases are generally not as regioselective as transferases but, with the correct combination of enzyme and substrate, single isomers can be formed. A recent example is the synthesis of the α-galactosyl epitope, which was synthesized from *N*-acetyllactosamine and α-*p*-nitrophenylgalactoside in 48% yield. A further improvement of the method is the conversion of glycosidases to glycosynthases by sitedirected mutagenesis. The application of method developed by Fort *et al.* (2000) yielded a highly efficient mutant cellulase that was used for β-1-4-linked oligosaccharides and polysaccharides. An interesting modification of reaction conditions to drive the glycosylation reaction forward proposed by Matsumura *et al.* (1999) stated the use of supercritical carbon

dioxide as a solvent, which has been applied to the synthesis of octyl-xylobiosides and xylotriosides.

Glycosyltransferases are used in nature to catalyse the formation of glycosidic bonds in high yields with excellent selectivity. As catalytic agents, they tend to be less readily available and more expensive than the glycosidases; however, they are more useful for glycosidic linkages that are difficult to make by other means such as the β-mannosidic linkage or α-sialosides (Koeller and Wong, 2000). Glycosyltransferases are also very useful for the efficient synthesis of larger oligosaccharides and glycoconjugates from chemically synthesised building blocks. Two recent examples are the chemoenzymatic synthesis of 7 (Koeller and Wong, 2000) and 8 (Depre *et al.*, 1999) using fucosyl- and sialyltransferases in the last step of the synthesis after final deprotection of the chemically synthesised core saccharides. The macromolecular nature of their natural substrates makes glycosyltransferases also ideal enzymes for oligosaccharide synthesis on the solid phase (Sallas and Nishimura, 2000). The repertoire of reactions that can be catalyzed by glycosyltransferases and glycosidases is ever increasing, spurred on by their biological relevance and their use in *in vitro* synthesis; therefore, more and more enzymes are being isolated and over expressed heterologously. Seto *et al.* (2000) aided the applications of mutagenesis methods on naturally occurring transferases to modify their substrate specificity.

2.4. β-Glucosidase

β-Glucosidase (β-D-glucoside glucohydrolases; EC 3.2.1.21) catalyzes hydrolysis of a wide range of β-glucosides including alkyl-, aryl- β-glucosides, diglucosides, oligosaccharides etc. In general, glucosidases encompass a heterogeneous group of enzymes cleaving β 1-4 linkages of di- and oligo-saccharides, or other gluco conjugates with hetero molecules (aglycones) and occurs ubiquitously in eukaryotes, eubacteria and archaea (Woodward and

Wiseman, 1982).

β-Glucosidases have been classified according to various criteria and thus there is no single well-defined method for the classification of these versatile enzymes. Henrissat (1996) exposed two bases for the classification of β-glucosidases, (i) substrate specificity, and (ii) nucleotide sequence identity. One of the first classification schemes based on the available sequences proposed grouping of these enzymes into two types, namely, Type I and Type II β-glucosidases. Another proposed scheme divided β-glucosidases into two subfamilies, subfamily A (BGA) and subfamily B (BGB). However, a classification based on substrate specificity states about -aryl-β-glucosidases, -true cellobiosiases, and -broad susbtrate specificity enzymes classes which are also active on a wide array of substrates. These enzymes display broad substrate specificity with respect to the aglycone portion of the substrate. Numerous studies have provided insight into the substrate specificity of β-glucosidases isolated from different organisms wherein, most of the family 1 enzymes show significant β-galactosidases activity. β-Glycosidases have been classified into one of the 57 families of glycosidases, and β-glucosidases are classified as Family 1 glycosidases based on amino acid sequence similarity and substrate specificity. A remarkable feature of the enzymes belonging to this family is a wide range of substrate preferences despite high sequence homology (Henrissat and Romeus, 1995; Henrissat, 1996).

In brief, the classification scheme proposed for all glycosylhydrolases (nearly 2000 in number) resulted in recognition of 88 families. β-Glucosidases with the exception of glucosylceramidase (acid β-glucosidase), a memebr of family of 30, are placed in either family 1 or family 3 of glycosylhydrolases. Family 1 comprises nearly 62 β-glucosidases from archaebacteria, plants, mammals, and also includes 6-phosphoglycosidases and thioglucosidases. Most family 1 enzymes also show significant β-galactosidase activity. The family 1 β-glucosidases are also classified as members of the 4/7 super family with a

common eight-fold β/α barrel motif. Here, the active site is placed in a wide cavity defined along the axis of the barrel, with a putative acid/base catalyst located at the end of β-strand 4 and a catalytic nucleophile near the end of β-strand 7. The 4/7 super family also includes other enzymes like family 2 β-galactosidase, family 5 cellulases, family 10 xylanase, and family 17 barley glucanases. Family 3 of glycosylhydrolases consists of nearly 44 β-glucosidases and hexosaminidases of bacterial, mold, and yeast origin (Bhatia *et al.*, 2002).

2.4.1. β-Glucosidases from microbes

Microbial β-glucosidases have been the subject of extensive research and have given a lot of promising information regarding the functions and characters of the enzyme. The cellulolytic enzymes are composed of three main activities: endoglucanase, exoglucanase and β-glucosidase, are wide spread among bacterial and fungal strains even though the exoglucanases (also called cellobiohydrolases) are rare in the bacterial kingdom. More than hundred of endo and exoglucanase sequences are known. Cellulolytic enzymes are also known to play an important role in some industrial applications, such as in bio-stone washing of jeans, replacing the stones in their abrasive effect on the garments. β-Glucosidases are also important in the regulation of cellulase genes since they are the key enzyme in the synthesis of sophorose, an efficient inducer of the cellulolytic system of *Trichoderma reesei* (Bhatia *et al.*, 2002). They constitute also the focus of many applied researches since they are not only needed in the cellulose breakdown but also in the synthesis of oligomers and other complex molecules (such as alkyl-glucosides) by transglycosylation (Bhatia *et al.*, 2002).

One of the earliest bacterial β-glucosidase, purified from *Agrobacterium faecalis* by Day and Withers (1986) was a dimmer of 50 kDa monomer and exhibited high specificity for cellobiose (Trimbur *et al.*, 1992). The fungal enzymes are used in several biotechnological processes, including development of novel carbohydrate foods, alcohol based fuels and other commercial products from

cellulose. Particularly, glucose production can be achieved from the most abundant biological macromolecule, cellulose, by the extracellular enzyme complex (cellulase) that is derived from various fungal species such as *Trichoderma.* A cellulase complex isolated by Fowler (1993) from *Trichoderma reseei* comprised at least three different enzymes that together hydrolyze cellulose to oligosaccharides and glucose. Of these, the endoglucanases and cellobiohydrolases synergistically hydrolyze cellulose into small cellooligosaccharides, mainly cellobiose.

Another major application of microbial β-glucosidases is in the flavor and fragrance industry. Gueguen *et al.* (1996) reported that β-glucosidases are key enzymes in the release of aromatic compounds from glucosidic precursors present in fruits and fermentating products. Indeed, many natural flavor compounds, such as monoterpenols, C-13 norisoprenoids, and shikimate-derived four compounds, accumulate in fruits as flavorless precursors linked to mono- or diglycosides and require liberation by enzymatic or acidic hydrolysis (Vasserot *et al.*, 1995; Winterhalter and Skouroumonis, 1997). Microbial β-glucosidases can also be used to improve the organoleptic properties of citrus fruit juices, in which the bitterness is in part due to a glucosidic compound. The studies by Gunata *et al.* (1985) showed that monoterpenols in grapes (e.g., linalol, geraniol, nerol, citronelol, α-terpineol and linalol oxide) are linked to diglucosides, which contribute to the flavor of wine. The enzymatic hydrolysis of these compounds requires a sequential reaction, which produce monoglucosides. Subsequently, monoglucosides are hydrolyzed by the action of β-glucosidases. Endogeneous β-glucosidases from grape are not sufficient to process the hydrolysis of monoterpenyl-glucosides. The grape enzymes display limited activity towards these glucosides and a large fraction of the aromatic compounds remains unprocessed in mature fruit. The addition of glucose-tolerant exogenous β-glucosidase isolated from fungi (*Aspergillus oryzae*) was shown to improve the

hydrolysis of glucoconjugated aromatic compounds and enhance wine quality (Riou *et al.*, 1998).

Enzymes derived from thermophillic microorganisms are often preferred for the transformation of lactose in milk or whey because of their various advantages such as increased thermostability, reduction in growth of unwanted microbial contaminants at elevated temperatures, and reduction in viscosity of the reaction system. An intracellular β-glycoside hydrolase with β-glucosidase and β-galactosidase activity was isolated by Nakkharat and Haltrich (2006) from the thermophilic ascomycete *Talaromyces thermophilus*. Amouri and Gargouri (2006) reported an improved mutant cellulolytic fungal (*Stachybotrys* sp.) β-glucosidase which showed activity towards salicin, methyl-umbellypheryl-β-S-glucoside and *p*-nitrophenyl-β-D-glucoside, thus it showed a true β-glucosidase activity since it splits cellobiose into two glucose monomers. The enzyme showed more affinity to *p*NPG than cellobiose and salicin. Another inducible mycelial β-glucosidase from *Scytalidum thermophilum* reported by Zanoelo *et al.* (2004) showed wide sustrate specificity and was activated by glucose or xylose which is a distinguished character of this enzyme from all others. Like *Scytalidium*, an extracellular β-glucosidase from wooddecaying fungus *Daldinia eschscholzii* showed *p*-NP-β-D-glucopyranoside specificity and was competitively inhibited by glucose. The internal amino acid sequences of *D. eschscholzii* β-glucosidase have similarity to the sequences of the family 3 β-glucosyl hydrolase (Karnchanatat *et al.*, 2007).

Microbial β-glucosidases are vastly implicated in biotechnological application and have been well-characterized. Many of microbial β-glucosidases are reported to their physico-kinetic level characterization which will be used to highlight the present comparative study.

2.4.2. β-Glucosidases from animals

In animals especially humans, several β-glucosidases have been described for their physiological role in metabolism. For example, the lysosomal β-glucosidase (acid β-glucosidase) hydrolyzes glucocerebrosides (glycosphingolipids) present in the lysosomal membranes, and a lack of this enzyme is the cause of the various forms of Gaucher's disease, one of the hereditary lysosomal storage disorders. A membrane associated glucocerobrosidase (EC 3.2.1.45) reported by Beutler (1992) was weakly glycosylated enzyme in lysosome which cleaved the substrate glucosylceramide (glucocerebroside) to ceramide and glucose. Lysosomal β-glucosidase is present in most tissues and cell types with various levels of catalytic activity. A deficiency of the lysosomal β-glucosidase in humans results in a condition known as Gaucher disease. Seven different mutations in human β-glucosidase have shown to result in inactive enzymes (Ohashi *et al.*, 1992).

Cytosolic β-glucosidases are mainly present in the liver, kidney and intestine and are reported to play a key role in detoxification of plant β-glucosides which was based on the broad specificity towards aglycone moieties of mono and disaccharide substrates such as L-picein, salicin, arbutin, amygdalin, prunasin, visine and linamarin that are found in plants consumed by animals. The study on first heterologous expression of human CBG was accomplished by Berrin *et al.* (2002). A full-length CBG cDNA (*cbg-1*) was cloned from a human liver cDNA library and expressed in the methylotrophic yeast *Pichia pastoris*. It has been reported that the human enzyme had significant activity towards many common dietary xenobiotics including glycosides of phytoestrogens, flavonoids, simple phenolics and cyanogens with higher apparent afinities for dietary xenobiotics than for other aryl-glycosides. These data indicated that human CBG hydrolyzed a broad range of dietary glucosides and may play a critical role in xenobiotic metabolism (Berrin *et al.*, 2002). In a recent study, Tribolo *et al.* (2007) advocated that human cytosolic β-glucosidase (hCBG) was a xenobiotic-

metabolizing enzyme that hydrolyzes certain flavonoid glucosides, with specificity depending on the aglycone moiety, the type of sugar and the linkage between them.

A putative protein, predicted from the klotho (kl) gene showed homology to family 1 glycosyl hydrolase and was also predicted to occur in the cytosol of certain human cells where it might have a role in human aging (Kuro *et al.*, 1997). Another human β-glucosidase was specific for the hydrolysis of pyridoxine 5′-β-D-glucopyranoside, a common dietary form of vitamin B6, and has been ascribed a role in vitamin B6 bioavailability (McMahon *et al.*, 1997).

A β-glucosidase with broad regiospecific activity from China white jade snail (*Achatina fulica*) was found to cleave both β-(1→2)-glucosidic linkage at 3-C and β-(1→6)-glucosidic linkage at 20-C of ginsenosides and can hydrolyze ginsenosides Rb_1, Rb_2, Rb_3 and Rc. The enzyme specifically hydrolyzed the β-D-glucosides involving aryl-β-glucosides, alkyl-β-glucosides, and β-linked disaccharides (i.e. sophorose, gentiobiose, and cellobiose) (Hu *et al.*, 2007). Insecta have shown interesting β-glucosidase system; a β-glucosidases from the ventriculus and honey sac and the hypopharyngeal glands of *Apis mellifera* reported by Pontoh and Low (2002) were glycoprotein.

Abstracting from detailed studies on animal β-glucosidase protein and nucleic acid researches it appears that the β-glucosidase will provide the basis for future studies on the physiological role of this enzyme.

2.4.3. β-Glucosidases from plant kingdom

β-Glucosidase has occupied a varity of roles and has been well characterized for its significant metabolic functionality in plants biological processes and potential biotechnological applications. It plays an important role in defense against some pathogens and herbivores by releasing hydroxamic acids, coumarins, thiocyanates, terpenes, and cyanide from the corresponding glucosides (Fenwick *et al.*, 1983; Jones, 1988; Niemeyer, 1988; Poulton, 1990;

Oxtoby *et al.*, 1991). Plant β-glucosidases also function in the hydrolysis of conjugated phytohormones (i.e., glucosides of giberellins, auxins, abscisic acid, and cytokinins) (Wiese and Grambow, 1986; Brzobohaty *et al.*, 1993). One of the finest implicit functions of β-glucosidases in plants is the hydrolysis of the cyanogenic glucosides (cyanogenesis). In this case, the enzyme and substrate occur in different subcellular compartments and come into contact with each other only after tissue disruption by pathogens or herbivores. As a result, the cyanogenic glucosides are hydrolyzed, releasing toxic hydrogen cyanide (HCN). Many economically-important crops, including sorghum, cassava, lima beans and cherries accumulate cyanogenic glucosides and the hydrolysis of these compounds produces the respiratory poison HCN (Conn, 1981; Jones, 1988; Poulton, 1988; Oxtoby *et al.*, 1991). The studies on white clover (*Trifolium repens* L) by Hughes (1993) revelaed two related cyanogenic glucosides (linamarin and lotaustralin) were the substrates of linamarase (β-glucosidase), which is a glycosylated protein having high-mannose-type N-asparagin-linked oligosaccharides which hydrolyze to glucose and HCN. Linamarases are localized the cell walls of the epidermal cells of leaves (Kakes, 1985; 1993). Other cyanogenic linamarase from cassava has been studied in detail by various researchers (Mkpong *et al.*, 1990; Yeoh and Woo, 1992; Hughes, 1993). The cassava plant is a staple food in most African countries which is a highly cyanogenic plant and causes acute cyanide poisoning in humans (Poulton, 1989). Hosel *et al.* (1987) have reported two dhurrinases (Dhurrinase 1 and Dhurrinase 2) from *Sorghum* seedling which shared a number of characteristics feature with maize and plastid localization (Esen and Stetler, 1993).

Bennett *et al.* (1997) reported a different type, dicot β-glucosidase (myrosinase; thioglucoside glucohydrolase, EC 3.2.3.1)) from family *Brassicaceae* catalyzed the hydrolysis of secondary compounds, glucosinolates (thioglucosides), which contained a glucose residue with a sulfur- and nitrogen-containing side chain. In oilseed rape three structural classes of glucosinolates,

derived from phenylalanine (aromatic), methionine (aliphatic/alkenyl) and tryptophan (indolyl) were reported by Fenwick *et al.* (1983). Glucosinolates are stored in the vacuoles of plants and when the tissue is disrupted they are converted by myrosinase to toxic compounds such as isothiocyanates, thiocyanates, and nitriles. The resulting byproducts of the reaction cause thyroid and liver disease in animals and humans who consume glucosinolate-rich foods such as rape (*Brassica napus*). This enzyme-substrate system is implicated as a defense system against herbivorous insects, slugs, and fungal pathogens as well as being involved in host-plant recognition by specialized insect pests of oilseed rape (Fenwick *et al.*, 1983). Falk and Rask (1999) reported a β-glucosidase from seeds of *Brassica napus* (oilseed rape) had 130 kDa native molecular mass consisted of a disulfide linked dimer of 64 kDa monomers and the internal amino acid sequences were used in cloning of cDNA for the enzyme. One nearly full-length and one partia1 β-glucosidase-encoding cDNA clone were isolated and sequenced. Zeatin-*O*-glucoside was identified as a natural substrate for *B. napus* β-glucosidase.

A detailed study on maize (*Zea mays* L.) β-glucosidase has been carried out by Esen (1992). Cuevas *et al.* (1992) reported that maize β-glucosidase cleaves hydroxamic acid glucosides (HxGlc), particularly 2-O-β-*D*-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMMBOAGlc). The aglycone DIMBOA is present as a glucoconjugate (DIMBOAGlc) in intact tissues of maize, rye and wheat. DIMBOA is a potent, toxic aglycone that has been shown to inhibit the electron transport system and phosphorylation reactions in bovine mitochondria and spinach chloroplasts (Niemeyer *et al.*, 1986).

Another plasticidal β-glucosidae from oat (*Avena sativa*) reported by Gus-Mayer *et al.* (1994) was found to be involved in the defense mechanism against fungal infection, destruction of the oat cell wall and plasma membrane, elicited by fungal infection, brings the plastidal β-glucosidase in contact with the vacuolar avenacosides. Consequently, the enzyme hydrolyzed inactive substrates

into active 26-desglucoavenacosides, which has been shown to possess anti-fungal activity. Based on the nucleotide sequence, the oat β-glucosidase is classified as a member of the family 1 glycosyl hydrolase (Gus-Mayer *et al.*, 1994). Minami *et al.* (1999) isolated a plasticidal β-glucosidase from leaves of the indigo plant (*Polygonum tinctorium*) which contained high activity for the substrate indican.

A germinating rice seed β-glucosidase ionically bound to cell walls was purified by Akiyama *et al.* (1998) and shown to have an apparent M_r 56 kDa with its N-terminal amino acid sequence (44 residues) exhibiting a high homology with those of β-glucosidases from other plants, such as barley and white clover. The enzyme showed hydrolytic as well as transglycosylation activity towards (1→3)-β- and (1→4)-β-linked oligosaccharides. This suggested that the β-glucosidase is probably involved not only in hydrolysis but also in modification of oligosaccharides in cell walls of germinating rice seeds (Akiyama *et al.*, 1998). In another study Opassiri *et al.* (2003) reported that the cDNAs for two β-glucosidase isozymes from rice (*Oryza sativa* L.), designated *bglu1* and *bglu2* were cloned and sequenced which included open reading frames encoding 504 and 500 amino acid precursor proteins, respectively. However, differences were seen in expression in mature plants, where *bglu1* was highly expressed in flowers, but *bglu2* was not.

A β-glucosidase from rye (*Secale cereale*) shoots reported by Sue *et al.* (2000) was highly active not only on DIMBOA-Glc but also on its 7-demethoxy analogue as substrate, DIBOA-Glc-, a substrate specificity different from those of maize and wheat glucosidases. The wheat (*Triticum aestivum*) and rye (*Secale cereale*) β-D-glucosidases have been shown to hydrolyze hydroxamic acid-glucose conjugates and one distinctive property of the wheat and rye glucosidases is that they function as hexamers but lose activity when dissociated into smaller oligomers or monomers (Sue *et al.*, 2006). Similarly, a glycosidase containing β-glucosidase and β-fucosidase activities from *Dalbergia cochinsinensis* Pierre (Thai

Rosewood) has been reported by Srisomsap *et al.* (1996) to be 66 kDa pentahomodimeric subunit strucuture of 330 kDa native protein.

There are more than 120,000 secondary metabolites known to occur in higher plants and many of them are important because of various pharmacological and therapeutic applications (Gerasimenko *et al.*, 2002). Warzecha *et al.* (1999) studied the plant cell suspension cultures of *Rauwolfia* which contained a 61 kDa raucaffricine-*O*-β-D-glucosidase was found to be a new member of the family 1 of glycosyl hydrolases. Plants of *Rauvolfia serpentina* accumulate ajmaline as a major alkaloid, whereas cell suspension cultures of *Rauvolfia* mainly accumulate the glucoalkaloid raucaffricine. Cell cultures do contain a specific glucosidase known as raucaffricine-*O*-β-D-glucosidase (RG), which catalyzes the *in vitro* formation of vomilenine, a direct intermediate in ajmaline biosynthesis. Zárate *et al.* (2001) established different transgenic cell lines of *Nicotiana tabacum* expressing strictosidine β-glucosidase cDNA from *Catharanthus roseus* following *Agrobacterium tumefaciens* infection. Molecular data showed that *C. roseus* cells SGD activity was associated with a protein aggregate of a size of 650 kDa, and this was absent in control and samples of the transgenic lines which failed to show SGD activity (Zárate *et al.*, 2001).

Among several fruit seeds, apple seed was identified as a new promising β-glucosidase source for alkyl *O*-glucoside synthesis by reverse hydrolysis, since it showed high hydrolytic activities on a broad spectrum of β-glucosides. From the apple seed meal, a major glucosidase isoenzyme reported by Yu *et al.* (2007) showed higher thermal stability than β-glucosidase from almond preserved at 50 °C in an aqueous environment.

Winemaking is a biotechnological process in which the use of exogenous enzyme preparations helps to overcome the problem of the insufficient activity of endogenous activity in the grapes. The use of the enzymes in the wine industry remains limited for several reasons *viz.* traditionalism of winemakers, influence on enzymatic activities related to physicochemical characteristics of musts and

wines (pH, temperature, ethanol, sugars, polyphenols, etc.) on enzymatic activities. The major enzyme groups in winemaking are oxidoreductase, pectinase, protease and finally β-glucosidase. Any study of β-glucosidase enzymes must address the terpene compounds that contribute to the varietal character of wines. Reports indicate that not all glycosides are present in all grape varieties, and that concentrations vary according to variety (Gunata *et al.*, 1985). Major precursors include structures such as β-D-glucopyranoside, 6-*O*-α-L-arabinosyl-β-D-glucopyranoside, 6-*O*-α-L-ramnopyranosyl-β-D-glucopyranoside and 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside apiosylglycosides. Studies by Villena *et al.* (2007) confirmed that aglycon chemical structure (volatile when free) may vary, taking the form of a terpenol (linalol, geraniol, nerol, citronelol, hotrienol or α-terpienol), linalol oxide, linear or cyclic alcohol (hexanol, phenylethanol, benzyl alcohol), C_{13} norisoprenoid, phenolic acid and/or volatile phenol.

Dignum *et al.* (2004) reported a β-glucosidase from green vanilla (*Vanilla planifolia*) beans which showed activity towards eight naturally occurring glucosides in vanilla and towards *p*-nitrophenol. The enzyme does not have high substrate specificity for the naturally occurring glucosides compared to the synthetic *p*-nitrophenol glucoside. A high specificity β-glucosidase active on isoflavone conjugates from soybean (*Glycine max*) roots was highly activity against isoflavone conjugates. The enzyme was inactive against tested flavonol glycosides and it was supposed that this enzyme is involved in the release of daidzein and genistein, both of which play central roles in soybean defense (Hsieh and Graham, 2001). Anthocyanin content in Sicilian sweet orange (*Citrus sinensis* (L) Osbeck) varieties known as blood oranges (*Tarocco, Moroe sanguinello*) undergoes changes during the ripening process. The anthocyanin concentration reaches to maximum in the fully ripe fruit. At latter stage of maturity, a decrease of these pigments is observed. β-Glucosidase activity in *Tarocco* variety, the most

common Sicilian blood orange was estimated in order to underline its role on anthocyanins degradation during ripening (Barbagallo *et al.*, 2007).

Thus, β-glucosidase occurs ubiquitously in living organisms belonging to all kingdoms but performs varied functions in them. Plant β-glucosidases are known to function in chemical defense of young plant parts against pests by catalyzing the hydrolysis of toxic glucosides. β-Glucosidases accounts for the liberation of the flavoring compounds collectively called terpenols (nerol, geraniol, linalool), benzyl and phenylethyl alcohols from their respective glucoside precursors. Additionally, its functions in plants include the hydrolysis of phytohormone precursors, pigment metabolism, seed development, and biomass conversion.

2.4.4. Substrate specificity of β-glucosidases

β-Glucosidases exhibit broad specificity with respect to both the aglycone and the glycone moieties of their substrates. In fact, true β-glucosidases from all sources have a similar specificity for the glycone (glucose) portion of the glucoside; however they vary with respect to aglycone specificity. The cyanogenic diglycoside (R)-amygdalin (the gentiobioside of (R)-mandelonitrile) that accumulates in black cherry seeds and other stone fruits contains a disaccharide as the glycone part of the substrate (Poulton, 1993). Studies by Svasti *et al.* (1999) on an isoflavonoid glucoside from the seeds of Thai Rosewood (*Dalbergia cochinchinensis* Pierre) showed high specificity to 12-dihydroamorphigenin, dalcochinin. Dalcochinin-8′-*O*-β-D-glucoside is hydrolyzed by Thai Rosewood β-glucosidase, but not by other β-glucosidases, such as cassava linamarase, almond β-glucosidase, or mustard seed myrosinase. Similarily, a β-glucosidase from *Vanilla planifolia* reported by Dignum *et al.* (2004) was found to be involved in aroma generation expressed its highest affinity for a glucoside with a polar group in the *para*-position (*p*-nitrophenol, vanillin, vanillic acid, *p*-hydroxybenzaldehyde, and ferulic acid). Glucosides with an apolar or no

substituent in the *para*-position and a methoxyl-group in *ortho*-position are hydrolyzed poorly (guaiacol and creosol). Compounds lacking a polar group in the *para*-position and a methoxyl-group in *ortho*-position (*p*-cresol and 2-phenylethanol) or that have the glucose attached to the sidechain (2-phenylethanol) are not hydrolyzed at all.

Srisomsap *et al.* (1996) exposed an enzyme containing β-glucosidase and β-fucosidase activity from *Dalbergia cochinchinensis* Pierre which showed high efficacy towards *p*-NP-β-D-glucoside and a slow hydrolysis of *p*-NP-β-D-galactoside, *p*-NP-β-D-xyloside, and *p*-NP-α-L-arabinoside. Hydrolysis of sophorose, laminaribiose, and gentiobiose were slow and cellobiose hydrolysis was slowest reported. Cynogenic glucoside linamarin or prunasin were not hydrolyzed, but a little hydrolysis of amygdalin and salicin was recorded. Another β-glycosidase from the seeds of *Dalbergia nigescens* Kurz was reported by Chuankhayan *et al.* (2005) which showed its ability to hydrolyze *p*-nitrophenyl-β-D-glucoside and β-fucoside but this enzyme did not hydrolyze various glycosidic substrates efficiently and thus it was used to identify its own natural substrates. Two substrates were identified, isolated and their structures determined as: dalpatein 7-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside and 6,2′,4′,5′-tetramethoxy-7-hydroxy-7-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (dalnigrein 7-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside). The β-glycosidase removes the sugar from these glycosides as a disaccharide, despite its initial identification as a β-glucosidase and β-fucosidase. β-D-Glucan glucohydrolases can hydrolyze glycosidic linkages in several β-D--glucans, in β-D-oligoglucosides containing (1→2)-, (1→3)-, (1→4)-, or (1→6)-linkages, in aryl β-D-glucosides such as *p*-nitrophenyl-β-D-glucopyranoside, and in some β-D-oligoxyloglucosides. The barley β-D-glucan glucohydrolases reported by Hrmova *et al.* (1998) was also found to hydrolyze cyanogenic β-D-glucosides but with low activity. Their broad substrate specificity makes it difficult to assign these higher plant β-D-glucan glucohydrolases to current Enzyme Commission

classes; therefore, they have been described variously as β-D-glucan glucohydrolases, (1→3)-β-D-glucan exohydrolases, and β-D-glucosidases (Hrmova *et al.*, 1998; Varghese *et al.*, 1999).

Family 3 β-D-glucan glucohydrolases catalyze the hydrolytic removal of β-D-glucosyl residues from nonreducing termini of a range of β-D-glucans and β-D-oligoglucosides. Their broad specificity was explained by Hrmova *et al.* (2002) using x-ray crystallographic data obtained from a barley β-D-glucan glucohydrolase in complex with nonhydrolyzable S-glycoside substrate analogs and by molecular modeling of enzyme/substrate complexes. The glucosyl residue that occupies binding subsite -1 was locked tightly into a fixed position through extensive hydrogen bonding with six amino acid residues near the bottom of an active site pocket. In contrast, the glucosyl residue at subsite -1 was located between two Trp residues at the entrance of the pocket, where it is constrained less tightly. The relative flexibility of binding at subsite -1, coupled with the projection of the remainder of bound substrate away from the enzyme's surface, means that the overall active site can accommodate a range of substrates with variable spatial dispositions of adjacent β-D-glucosyl residues. The broad specificity for glycosidic linkage type enables the enzyme to perform diverse functions during plant development (Hrmova *et al.*, 2002).

Among glycoside hydrolases, β-glucosidase plays a unique role in many physiological and biocatalytical processes that involve the β-linked *O*-glycosyl bond of various oligomeric saccharides or glycosides. Structurally, the enzyme can be grouped into glycoside hydrolase family 1 and 3. Although the basic ("retaining, double-displacement") mechanism for the catalysis of family 3 β-glucosidase has been established, in-depth understanding of its structure–function relationship, particularly the substrate specificity that is of great interest for developing the enzyme as a versatile biocatalyst, remains limited. To further probe the active site, a comparative study was performed by Langston *et al.* (2006) on a family 3 β-glucosidase from *Aspergillus oryzae* with substrates and

competitive inhibitors of different structures, in attempt to evaluate the site-specific spatial and chemical interactions between a pyranosyl substrate and the enzyme. Plant β-glucosidases display varying substrate specificities; Verdoucq *et al.* (2003) reported that the maize β-glucosidase isozyme Glu1 (ZmGlu1) was found to hydrolyze a broad spectrum of substrates in addition to its natural substrate DIMBOA-Glc (2-*O*-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxaxin-3-one), whereas the sorghum β-glucosidase isozyme Dhr1 (SbDhr1) hydrolyzed exclusively its natural substrate dhurrin (*p*-hydroxy-(S)-mandelonitrile-β-D-glucoside). Structural data of enzyme-substrate and enzyme-aglycone complexes have showed that five amino acid residues (Phe^{198}, Phe^{205}, Trp^{378}, Phe^{466}, and Ala^{467}) are located in the aglycone-binding site of ZmGlu1 and form the basis of aglycone recognition and binding, hence substrate specificity. Verdoucq *et al.* (2003) studied the mechanism of substrate specificity further, mutant β-glucosidases were generated by replacing Phe^{198}, Phe^{205}, Asp^{261}, Met^{263}, Phe^{377}, Phe^{466}, Ala^{467}, and Phe^{473} of Glu1 by Dhr1 counterparts. The simple mutant replacing Phe^{198} by a valine had the most drastic effect on activity, because the capacity of this enzyme to hydrolyze β-glucosides was almost completely abolished. The analysis of this mutation was completed by a structural study of the double mutant ZmGlu1-$E^{191}D$, $F^{198}V$ in complex with the natural substrate. The structure reveals that the single mutation $F^{198}V$ caused a cascade of conformational changes, which were unpredictable by standard molecular modeling techniques. Some other mutations led to drastic effects: replacing Asp^{261} by an asparagine decreases the catalytic efficiency of this simple mutant by 75% although replacing Tyr^{473} by a phenylalanine increased its efficiency by 300% and also provided a new substrate specificity by hydrolyzing dhurrin (Verdoucq *et al.*, 2003).

2.4.5 Transglycosylation by glycosidases

β-Glucosidases normally catalyze the hydrolysis of β-glucosidic linkages between D-glucose and an aglycone or another sugar and are evolutionarily related. Like other glycosidases, some β-glucosidases may catalyze reverse hydrolysis and transglucosylation, leading to synthesis of oligosaccharides and alkyl glucosides. Particularily, in the presence of alcohols, alkyl glucosides may be formed by reverse hydrolysis using glucose or by transglucosylation using sugar donors such as *p*-NP-β-D-glucopyranoside or cellobiose. Under certain conditions, the reverse of hydrolysis, that is, synthesis of glycosyl-bond between different molecules can occur. This takes place *via* two different modes, reverse hydrolysis and transglycosylation. In the first approach, modification of reaction conditions such as lowering of water activity (a_w), trapping of product or high substrate concentration leads to a shift in the equilibrium of reaction toward synthesis. This reaction is under thermodynamic control. In transglycosylation approach, a preformed donor glycoside (e.g., a disaccharide or aryl-linked glucoside) is first hydrolyzed by the enzyme with the formation of an enzyme-glycosyl intermediate. This is then trapped by a nucleophile other than water (such as a monosaccharide, disaccharide, aryl-, amino-, or alkyl-alcohol or monoterpene alcohol) to yield a new elongated product (Bhatia *et al.*, 2002).

Synthesis of alkyl glucosides has mainly employed high catalytically efficient almond β-glucosidase as well as *Fusarium oxysporum* and *Pyrococcus furiosus* β-glucosidases (Svasti *et al.*, 2003). Srisomsap *et al.* (1996) reported a β-glucosidase (dalcochinase) from the seeds of Thai rosewood (*Dalbergia cochinchinensis* Pierre) which catalyzed reverse hydrolysis using glucose as substrate, yielding di- and tri-saccharides and can also transfer glucose from *p*-NP-β-D-glc to alkyl alcohols. However, like almond and *Fusarium* β-glucosidases, dalcochinase uses primary alcohols as acceptors better than secondary alcohols, and cannot use tertiary alcohols at all. The inability of β-glucosidases to synthesize tertiary alkyl glucosides by transglucosylation or by reverse

hydrolysis appears to be a general phenomenon found with all glycosidases. In a report, Svasti *et al.* (2003) explored the exceptional ability of the cyanogenic β-glucosidase (linamarase) from cassava (*Manihot esculenta* Crantz) to transfer glucose to tertiary alcohols, and compare its action to that of Thai rosewood and almond β-glucosidases. These were reported to synthesize alkyl glucosides by transglucosylating alkyl alcohols of chain length C1–C8. Cassava linamarase shows greater ability to transfer glucose from *p*-nitrophenyl-β-glucoside to secondary alcohol acceptors than other β-glucosidases, and is unique in being able to synthesize C4, C5, and C6 tertiary alkyl β-glucosides with high yields of 94%, 82%, and 56%, respectively. Cassava linamarase required *p*-NP-glycosides as donors and could not use mono- or di-saccharides as sugar donors in alkyl glucoside synthesis (Svasti *et al.*, 2003).

Hansson *et al.* (2001) evaluated five different β-glycosidases (*Pyrococcus furiosus* β-glucosidase, *Sulfolobus solfataricus* β-galactosidase, *Caldocellum saccharolyticum* β-glucosidase, almond β-glucosidase and *Escherichia coli* β-galactosidase) as transglycosylation catalysts in hexanol containing various amounts of water. All enzymes catalyzed both hydrolysis and transglycosylation of the glycosidic substrates (pentyl- and *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-D-galactoside). From the concentration ratio (alcohol/water) a decrease in ratio of the transglycosylation tohydrolysis ratio was expected with increasing water activity in the hexanol. However, for all enzymes tested the selectivity for the alcohol increased with increasing water activity. This counteracted the effect of higher water concentration and in most cases the transglycosylation/hydrolysis ratio increased with increasing water activity. On the other hand, in hexanol/water two-phase systems, hydrolysis has been shown to be by far dominating reaction even though the total activity increased for all enzymes. However, Hansson *et al.* (2001) found deviations in cases when the reactions became thermodynamically controlled: at high water contents secondary hydrolysis reduced the transglycosylation yields while higher

transglycosylation yields than predicted were obtained at low water activity in some cases using enzymes poorly selective for the alcohol.

In a detailed study, Hays *et al.* (1998) elaborated that cytosolic β-glucosidase (CBG) catalyzes a variety of transglycosylation reactions, which have been been shown with other glycosylhydrolases to function in synthetic and genetic regulatory pathways. The catalytic mechanism, substrate specificity, and transglycosylation acceptor specificity of guinea pig liver CBG was investigated by several methods. These studies indicated that CBG employed a two-step catalytic mechanism with the formation of a covalent enzyme-sugar intermediate and that CBG will transfer sugar residues to primary hydroxyls and equatorial but not axial C-4 hydroxyls of aldopyranosyl sugars. Further analyses revealed that for aldopyranosyl substrates, the activation energy barrier is affected most by the presence of a C-6 carbon and by the configuration of the C-2 hydroxyl, whereas the binding energy is affected modestly by the configuration and substituents at C-2, C-4, and C-5. These indicated that the transglycosylation activity of CBG derived from the formation of a covalently linked enzyme-sugar intermediate and that the specificity of CBG for transglycosylation reactions is different from its specificity for hydrolysis reactions. The enzyme hydrolyzed β-D-galactopyranosides, β-D-fucopyranosides, β-D-xylopyranosides, and α-L-arabinopyranosides, in addition to β-D-glucopyranosides. The enzyme also catalyzed transglycosylation reactions in which a sugar residue is transferred from a substrate molecule to an acceptor to form a new glycoside. These properties are consistent with the fact that CBG is a configuration-retaining glycosidase. Collectively, Hays *et al.* (1998) suggested that the catalytic mechanism of CBG consists of a double-displacement reaction involving the formation of a stable enzyme-sugar intermediate.

Transglucosylation activity of rice (*Oryza sativa* L.) βGlu1 has been ascertained by Opassiri *et al.* (2004) using its ability to transfer glucose from *p*NPG to pyridoxine (vitamin B6). There were five major transglucosylation

products, as expected, four of them were *p*NP derivatives with the same R_f values as products of the control containing only enzyme and *p*NPG. These transfer products were compared that one product had an R_f value equal to that of *p*NP-β-D-cellobioside, and another had an R_f equal to that of *p*NP-β-D-cellotrioside. After hydrolysis transglucosylation product was identified to be pyridoxine 5′-*O*-β-D-glucopyranoside (Opassiri *et al.*, 2004).

A high catalytic active almond β-glucosidase has been used in transglycosylation reactivity and the stability of almond β-glucosidase in five different organic media was evaluated by Kannan *et al.* (2004). Transglucosylation involving *p*-nitrophenyl β-D-glucopyranoside as donor and β-1-N-acetamido-D-glucopyranose, which is a glycosylasparagine mimic, as acceptor was explored under different reaction conditions using almond β-glucosidase and cloned *Pichia etchellsii* β-glucosidase II. Both enzymes catalyzed the formation of (1→3)- as well as (1→6)- regioisomeric disaccharides, the former being the major product in cloned β-glucosidase II reaction while the latter predominated in the almond enzyme catalyzed reaction. Use of β-1-N-acetamido-D-mannopyranose and β-1-N-acetamido-2-acetamido-2-deoxy-D-glucopyranose as acceptors in almond β-glucosidase catalyzed reactions, however, did not afford any disaccharide products revealing the high acceptor specificity of this enzyme (Kannan *et al.*, 2004).

The hydrolytic and transglucosidic reactions of the *Aspergillus niger* Family 3 β-glucosidase were characterized by Seidle and Huber (2005). Advanced analyses indicated have indicated that the substrates became transglucosidic acceptors when present at high concentrations. The study has also showed that gentiobiose forms by an intermolecular reaction of the C6 hydroxyl of Glc rather than an intramolecular reaction and that an equatorial orientation of the C2 hydroxyl, the presence of a C6 primary hydroxyl and β-linkages with oligosaccharide acceptors are important for acceptor reactivity (Seidle and Huber 2005).

2.4.6. Catalytic mechanism of β-glucosidase

All family 1 β-glucosidases share a general mechanism for the hydrolysis of the β-glycosidic linkage between an anomeric carbon and glycosidic oxygen. There are two steriochemically-different hydrolytic mechanisms proposed for cleavage of the β-linkage between the glycone and aglycone parts of a β-glucoside. These mechanisms differ with respect to retention and inversion at the anomeric center of the reduced sugar residue. The β-glucosidases, together with most cellulases and xylanases are known to hydrolyze the substrate while retaining the anomeric configuration of the sugar moiety (Sinnott, 1990; Clarke *et al.*, 1993). The retaining mechanism involves acidic catalysis that protonates the substrate and leads to formation of a transition state oxocarbonium ion. The orientation of the catalytic group is thought to be complementary to anomeric configuration of sugar moiety. The acidic site (i.e. the carboxyl group) interacts with the site where glycosidic oxygen is found. At the end of the cleavage, the anomeric carbon of the monosaccharide reacts with a water molecule to generate β-D-glucopyranose. This mechanism closely resembles the double displacement mechanism proposed for lysozyme (Fig. 5) (Koshland, 1953; Clarke *et al.*, 1993). The inverting mechanism of the anomeric configuration is based on single displacement by a nucleophilic water molecule. The difference between retention and inversion of the anomeric configuration is that only one transition state occurs in the inversion step of the single displacement mechanism. The final product in the inverting mechanism is α-D-glucopyranoside (Sinnott, 1990). Withers and Street (1989) proposed the double displacement mechanism for β-glucosidase catalysis based on NMR studies. It has been elucidated that the substitution of hydroxyl group at C-2 of the sugar by electronegative fluorine destabilized the adjacent positive charge at the transition state (Withers and Street, 1989), resulting in decreased rates of glycosyl-enzyme formation and hydrolysis.

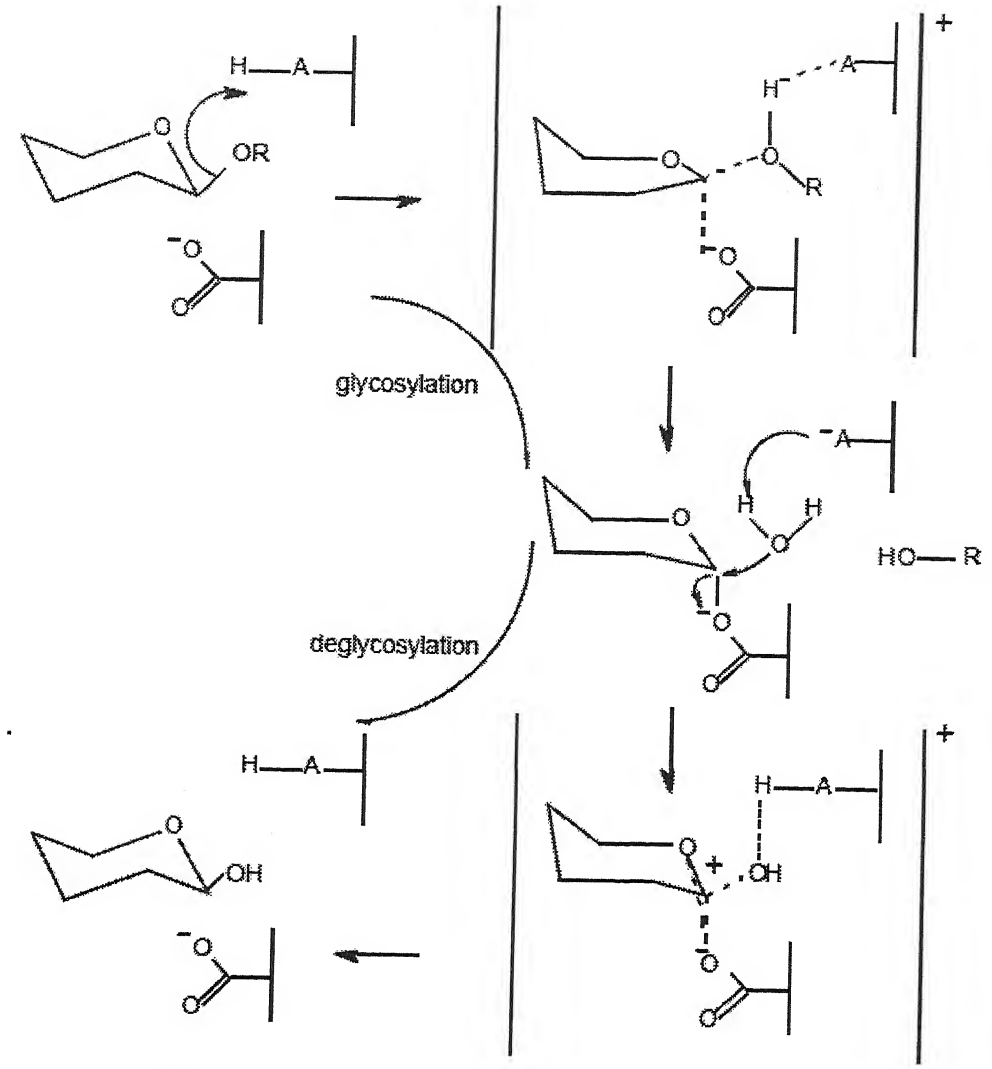


Figure 5: Proposed mechanism for hydrolysis of β-glycosidic bond by the "retaining" β-glycosidases (Wang and Withers, 1995).

This incorporation brought about considerable acceleration of glycosyl-enzyme formation without affecting the rate of glycosyl enzyme hydrolysis (Withers and Street, 1989). In contrast, the β-glycoside-fluorine resulted in retardation of deglycosylation step of the hydrolysis. The glycosyl enzyme intermediate was trapped easily in a transition state, thereby allowing the mechanism of β-glucosidase catalysis to be examined. Withers and Street (1989) also suggested

that a covalent glycosyl enzyme intermediate was formed during the formation of oxocarbonium ion in the transition state.

Formation of the enzyme-substrate complex in β-glucosidase catalyzed reaction is not well understood. Two pathways have been proposed for the transition state of β-glucosidase in the double displacement mechanism: *endocyclic* and *exocyclic* pathways. Both the pathways require two amino acid residues in the active site, one serving as a proton donor and the other as a nucleophile. In case of the exocyclic pathway, the carboxylic acid of the amino acid protonates the exocyclic oxygen (the glycosidic oxygen) of the substrate to make it a good leaving group. This gives rise to an unfavorable electronic geometry. The alternative pathway, endocyclic, involves protonation of the endocyclic oxygen in the formation of the enzyme-substrate complex (Clarke *et al.*, 1993). However, Withers and Street (1988) have suggested that these pathways may not be mutually exclusive and that β-glycosidases may act via either the endo or exocyclic pathway depending on the substrate. Once the transition state reached, β-glucosidases hydrolyzed the substrate while the configuration of the transition state was maintained.

In retaining and inverting mechanisms, at least two carboxyl groups are considered to participate in the catalysis of glycosyl hydrolases (Fig. 6). In inverting enzymes, these residues show an average distance of 9.3 Å. However, in retaining enzymes, these two residues are separated by 5.0 Å. In the retaining mechanism, the acidic group (glutamic acid) in the active site donates a proton to glycosidic oxygen and a nucleophilic group facilitates the bond breaking by attacking to C1 of glucose. Of these, the acid-base catalyst glutamic acid is in the motif TFNEP while the nucleophilic glutamic acid is in the motif I/VTENG.

Hydrolysis of the β-glycosidic bond releases the aglycone part and a water molecule provides a proton to a base catalyst (glutamic acid) and the OH^- group to the covalent bond between the glycone and the enzyme, releasing the glycone

and regenerating the nucleophilic glutamic acid. The active-site nucleophile has been identified using 2′-4′-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucopyronaside, which is a mechanism-based inhibitor of the enzyme (Withers *et al.*, 1990; Lawson *et al.*, 1996).

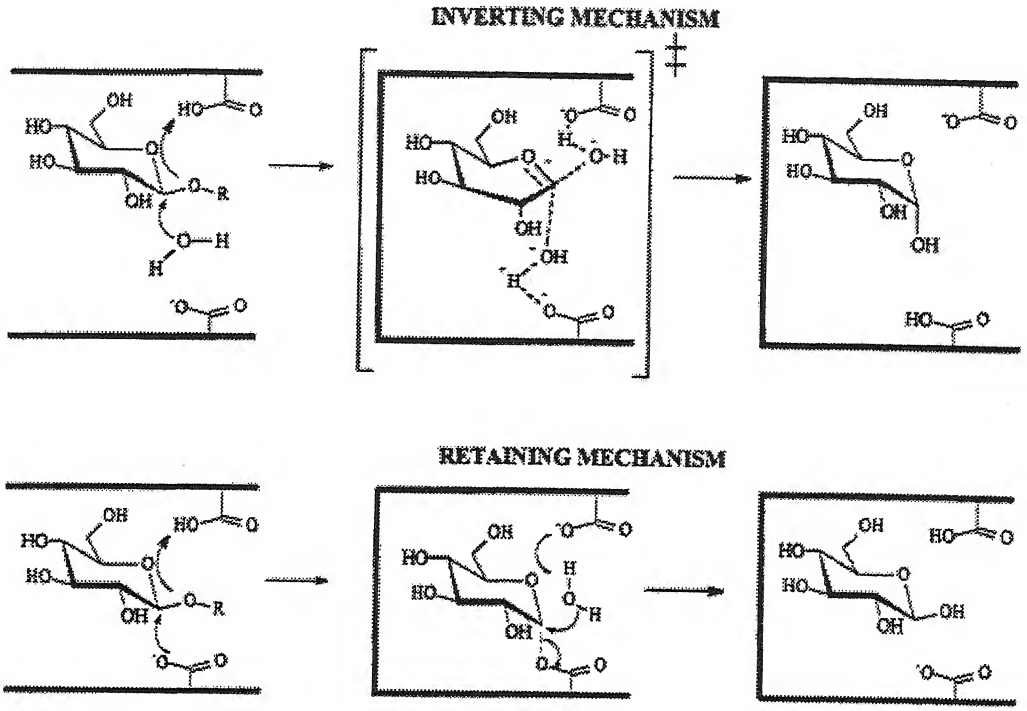


Figure 6: Mechanisms of retaining and inverting glycosidases (Sinnott, 1990).

Three-dimensional structures of several glycosyl–enzyme complexes have been determined through X-ray crystallographic analysis, revealing identities of important amino acid residues involved in the catalysis. In particular involvement of the carbonyl oxygen of the catalytic nucleophile in strong hydrogen bonding to the sugar 2-hydroxyl for the β-retainers or in interactions with the ring oxygen for α-retainers has been revealed by Withers (2001). The glucose ring in the "-1" (cleavage) site in the intermediates formed on several cellulases and a β-glucosidase adopts a normal $^4C_1$ chair conformation. By contrast the xylose ring at this site in a xylanase gets substantially distorted into a

$^{2,5}B$ boat conformation, a significant stereoelectronic implication. Substantial distortion has also been observed in the substrate upon binding to several β-glycosidases, this time to a $^{1}S_{3}$ skew boat conformation. Much less distortion is seen in the substrate bound on an α-transglycosylase. Finally, an efficient catalyst for synthesis, but not hydrolysis, of glycosidic bonds has been generated by mutation of the glutamic acid catalytic nucleophile of a β-glucosidase to an alanine. When used with α-glucosyl fluoride as a glycosyl donor, along with a suitable acceptor, oligosaccharides up to five sugars in length have been made with yields of up to 90% on individual steps (Withers, 2001).

2.5. Selected plants of the study

In this study, the medicinal plants were selected after some short of screening based on β-glucosidase enzyme profile and their possible glycosidic functional-relation. Consequently, the selected medicinal plants for the study are; *Withania somnifera* (Solanaceae), *Andrographis paniculata* (Acanthaceae), and *Silybum marianum* (Asteraceae). Brief reviews on these plants are given below.

2.5.1. *Withania somnifera* L. (Dunal)

Withania somnifera is a widely used plant in the traditional Ayurvedic medicine system of India, commonly know as "Ashwagandha" (or winter cherry). The plant belongs to the family "Solanaceae". *Withania* is an active ingredient in many formulations prescribed for a variety of musculoskeletal conditions (e.g., arthritis, rheumatism), and as a general tonic to increase energy, improve overall health and longevity, and prevent disease in athletes, aphrodisiac, the elderly, and during pregnancy (Chatterjee and Pakrashi, 1995). Many pharmacological studies have been conducted to investigate the properties of ashwagandha in an attempt to authenticate its use as a multi-purpose medicinal agent. In Ayurvedic medicine there is a class of herbs, including *Withania somnifera,* known as adaptogens or vitalizers. Adaptogens cause

adaptive reactions to disease, are useful in many unrelated illnesses, and appear to produce a state of nonspecific increased resistance (SNIR) to adverse effects of physical, chemical, and biological agents (Singh *et al.*, 1982).

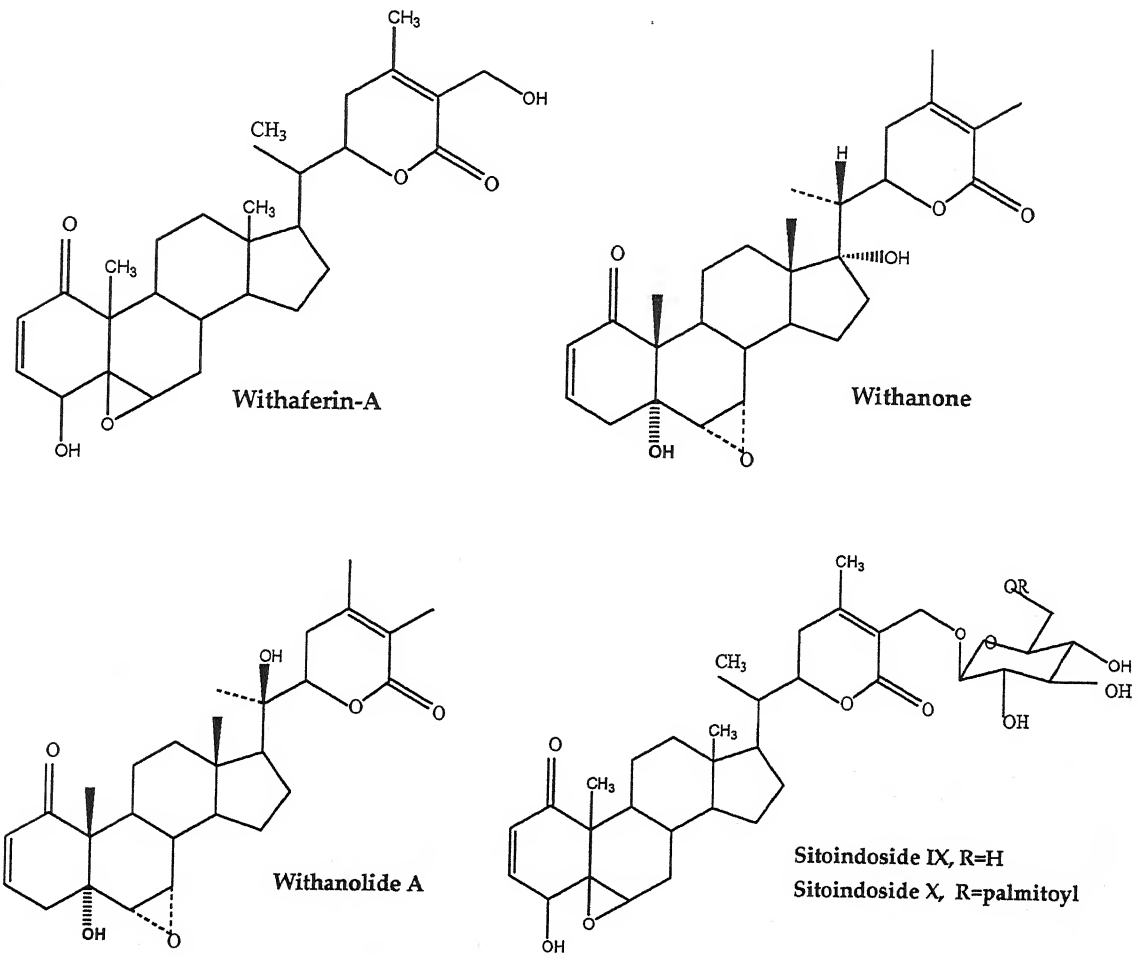


Figure 7: Major chemical constituents of *Withania somnifera.*

They are relatively innocuous, have no known specific mechanism of action, normalize pathological effects, and are usually glycosides or alkaloids of a plant. The chemistry of *Withania somnifera* has been extensively studied and over 35 chemical constituents have been identified, extracted, and isolated. The

biologically active chemical constituents are alkaloids (isopelletierine, anaferine), steroidal lactones (withanolides, withaferins), saponins containing an additional acyl group (sitoindoside VII and VIII), and withanolides with a glucose at carbon 27 (sitoindoside IX and X). Glycosides are the main compounds of interest regarding study on β-glucosidases, seven new withanolide glycosides called withanosides I, II, III, IV, V, VI, and VII from *Withania somnifera* were illucidated by Matsuda *et al.* (2001).

The effectiveness of ashwagandha in a variety of rheumatologic conditions may be due in part to its anti-inflammatory properties. *Withania somnifera* was found to cause considerable reduction in inflammation (Anbalagan and Sadique, 1981). Studies conducted on the mechanism of action for the anti-inflammatory properties on rats showed a decrease in absorption of ^{14}C-glucose in rat jejunum. Glucose absorption was maintained at the normal level by both *Withania somnifera* and the cyclooxygenase inhibitor oxyphenbutazone. Both drugs produced anti-inflammatory effects. Similar results were obtained in parallel experiments using ^{14}C-leucine absorption from the jejunum. These studies suggest cyclooxygenase inhibition may be involved in the mechanism of action of *Withania somnifera* (Somasundaram *et al.*, 1983). The use of *Withania somnifera* in treating various forms of cancer, the antitumor and radiosensitizing effects was investigated in urethane-induced lung adenomas in adult male albino mice (Singh *et al.*, 1986). *Withania somnifera* was also found to act as a radio- and heat sensitizer in mouse S-180 and in Ehrlich ascites carcinoma. Antitumor and radiosensitizing effects of withaferin (a steroidal lactone of *Withania somnifera*) were also seen in mouse Ehrlich ascites carcinoma *in vivo*. The studies of Devi *et al.* (1995) are suggestive of antitumor activity as well as enhancement of the effects of radiation by *Withania somnifera*.

Glycosides of *Withania somnifera* (sitoindosides VII and VIII) exhibited significant antistress activity in forced swimming induced immobility in mice; restraint stress induced gastric ulcers in rats, restraint-induced auto-analgesia in

rats, restraint stress effect on thermic response of morphine in rats, and morphine-induced toxicity in aggregated mice (Bhattacharya *et al.*, 1987).

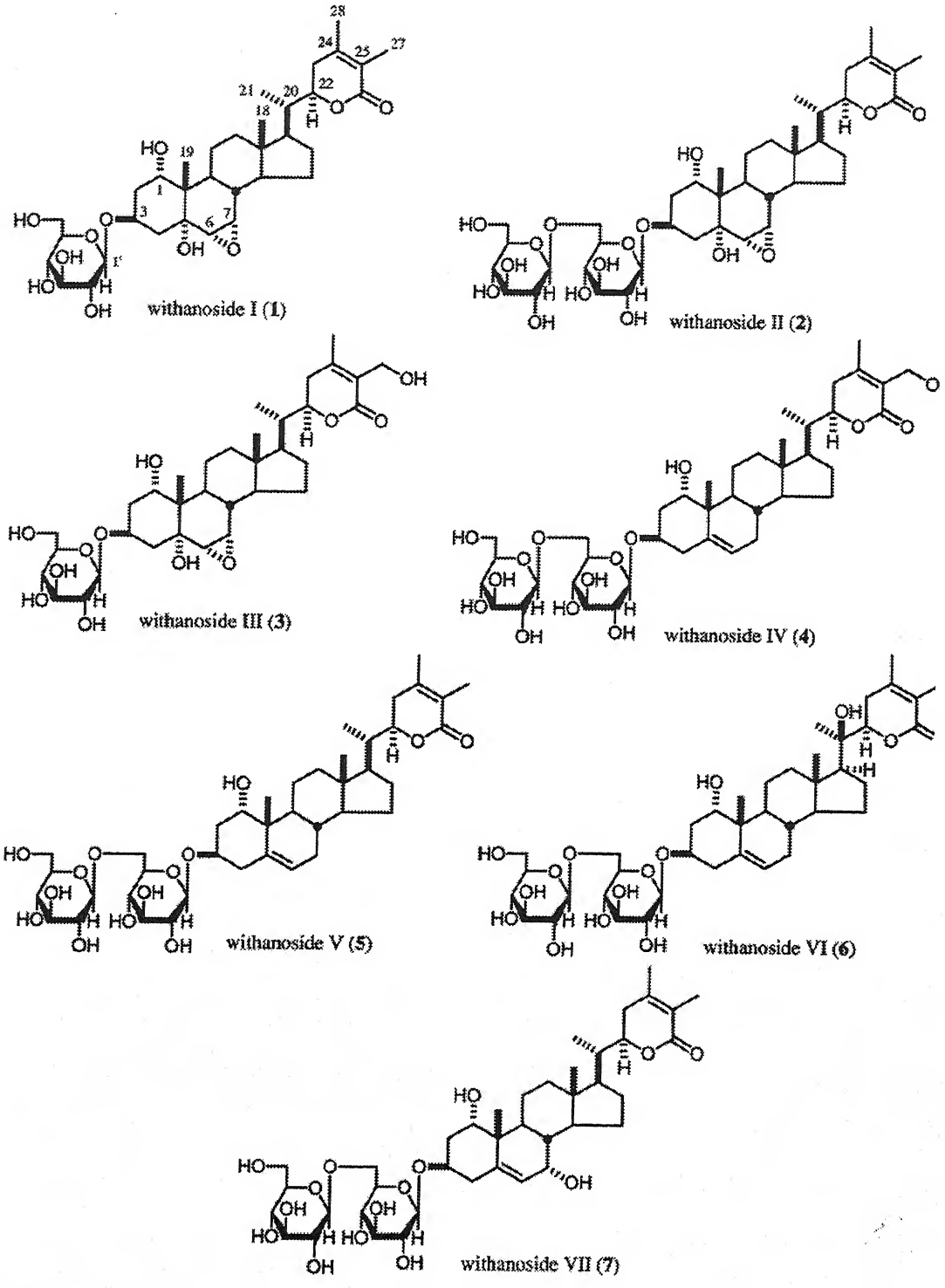


Figure 8: Structures of Withanosides deriveatives from *Withania somnifera.*

The brain and nervous system are relatively more susceptible to free radical damage than other tissues because they are rich in lipids and iron, both known to be important in generating reactive oxygen species. The brain also uses nearly 20% of the total oxygen supply. Free radical damage of nervous tissue may contribute to neuronal loss in cerebral ischemia and may be involved in normal aging and neurodegenerative diseases, e.g., epilepsy, schizophrenia, Parkinson's, Alzheimer's, and other diseases. Since traditional Ayurvedic use of *Withania somnifera* has included many diseases associated with free radical oxidative damage, it has been considered likely the effects may be due to a certain degree of antioxidant activity. The active principles of *Withania somnifera,* sitoindosides VII-X and withaferin-A (glycowithanolides), have expressed antioxidant activity using the major free-radical scavenging enzymes, superoxide dismutase, catalase, and glutathione peroxidase levels in the rat brain frontal cortex and striatum. Decreased activity of these enzymes leads to accumulation of toxic oxidative free radicals and resulting degenerative effects. This implies that *Withania somnifera* does have an antioxidant effect in the brain which may be responsible for its diverse pharmacological properties (Bhattacharya *et al.*, 1997). Total alkaloid extract of *Withania somnifera* roots has been hown its effects on the central nervous system. It exhibited a taming effect and a mild depressant (tranquilizer) effect on the central nervous system in monkeys, cats, dogs, albino rats, and mice. It had no analgesic activity in rats but increased Metrazol toxicity in rats and mice, amphetamine toxicity in mice, and produced hypothermia in mice. Effects of sitoindosides VII-X and withaferin from roots of *Withania* were studied on brain cholinergic, glutamatergic and GABAergic receptors in male Wistar rats (Schliebs *et al.*, 1997). Root extract of *Withania somnifera* was showed immunomodulatory effects in three myelosuppression models in mice: cyclophosphamide, azathioprin, or prednisolone. Significant increases in hemoglobin concentration, red blood cell count, white blood cell count, platelet count, and body weight were observed in WS-treated mice compared to

untreated control mice. Interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) production was also markedly decreased. The major activity of *Withania somnifera* may be the stimulation of stem cell proliferation. *Withania somnifera* reduced CTX-induced toxicity and may prove useful in cancer chemotherapy which needs to confirm the hemopoetic effect with other cytotoxic agents and to determine its usefulness as an adjuvant in cancer chemotherapy (Ziauddin *et al.*, 1996).

2.5.2. *Andrographis paniculata* (Burm. F.) Wall. Ex Nees

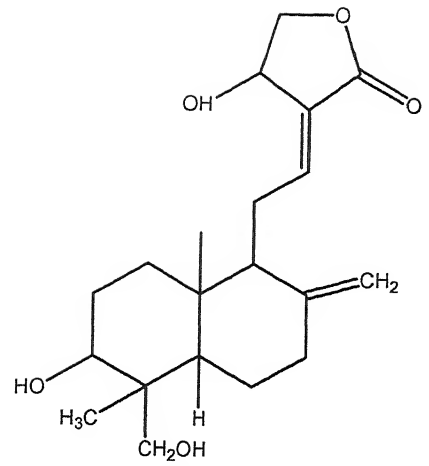
Andrographis paniculata (AP), the Kalmegh is commonly as "King of Bitters," belongs to the plant family Acanthaceae. It has been used for centuries in Asia to treat gastro-intestinal tract and upper respiratory infections, fever, herpes, sore throat, and a variety of other chronic and infectious diseases. It is evident from Indian Pharmacopoeia that it is a prominent member of at least 26 Ayurvedic formulations. Whereas in Traditional Chinese Medicine, *Andrographis* is an important "cold property" herb which is used to treat the heat of body in fevers, and to dispel toxins from the body and to prevent and treat common colds.

A number of diterpenoids and diterpenoid glycosides have been reported containing similar carbon skeleton, mainly andrographolide, neoandrographolide, deoxyandrographolide and several others are 14-deoxyandrographolide, 14-deoxy-11,12-didehydroandrographolide, andrographiside, deoxyandrographiside, homoandrographolide, andrographan, andrographon, andrographosterin and stigmasterol (Siripong *et al.*, 1992). The leaves of Andrographis contain the highest amount of andrographolide, the most medicinally active phytochemical in the plant, while the seeds contain the lowest (Sharma *et al.*, 1992). The structure of andrographolide contains (i) an a-alkylidene c-butyrolactone moiety, (ii) two olefin bonds Δ8(17) and Δ12(13), and (iii) three hydroxyls at C-3, C-19, and C-14. Of the three hydroxyl groups, the one

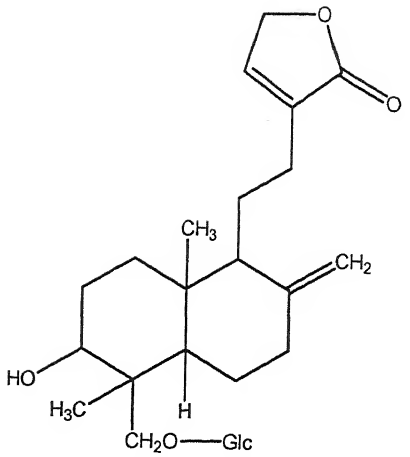
at C-14 is allylic in nature, and the others at C-3 and C-19 are secondary and primary, respectively. Matsuda *et. al.* (1994) revealed six new diterpenoids of ent-labdane type, 14-epi-andrographolide, isoandrographolide, 14-deoxy-12-methoxyandrographolide, 12-epi-14-deoxy-12-methoxyandrographolide, 14-deoxy-12-hydroxyandrographolide and 14-deoxy-11-hydroxyandrographolide as well as two new diterpene glucoside, 14-deoxy-11,12-didehydroandrographi-side and 6′-acetylneoandrographolide, and four new diterpene dimers, bisandrographolide A, B, C and D, were isolated along with six known compounds. Phytochemical investigation of the roots and aerial parts of *Andrographis paniculata* yielded a new flavone, 5-hydroxy-7,20,60-trimethoxyflavone and an unusual 23-carbon terpenoid, 14-deoxy-15-isopropylidene-11,12-didehydroandrographolide together with five known flavonoids and four known diterpenoids and one deoxyandrographolide-19β-D-glucoside.

Studies on mice showed that *Andrographis paniculata* is a potent stimulator of the immune system in two ways: (i) Antigen-specific response: antibodies are made to counteract invading microbes, and (ii) Nonspecific immunse response: macrophage cells scavenge and destroy invaders. AP activates both responses - making it effective against a variety of infectious and oncogenic (cancer-causing) agents (Puri *et al.*, 1993). The initial interest with AP has its hepatoprotective (liver protecting), as well as its anti-cancer properties; similarly, it has an effective treatment in immune boosting.

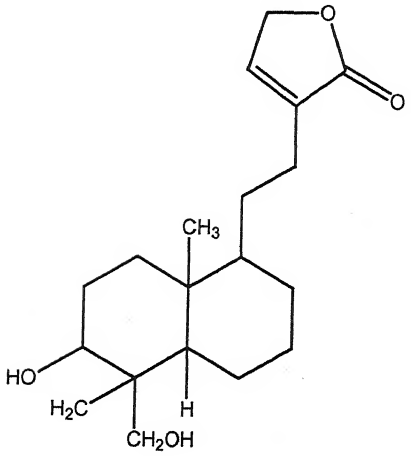
Andrographis have great promising effect on viability of the HIV virus; andrographolide prevented transmission of the virus to other cells and stopped the progress of the disease by modifying cellular signal transduction. Andrographolide probably does this by inhibiting enzymes that facilitate the transfer of phosphates. Andrographolide may inhibit HIV-induced cell cycle dysregulation, leading to a rise in $CD4^+$ lymphocyte levels in HIV-1 infected individuals (Holt *et al.*, 1998).



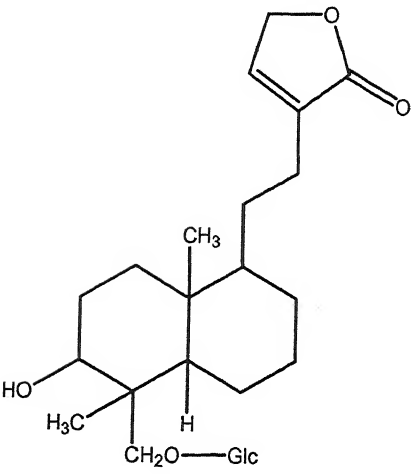
Andrographolide



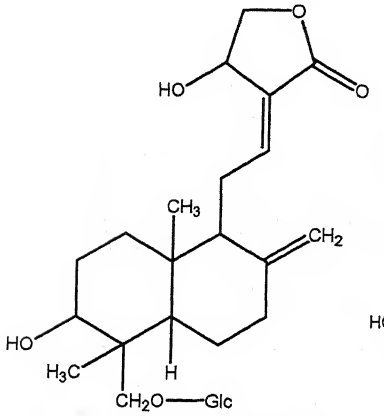
Neoandrographolide



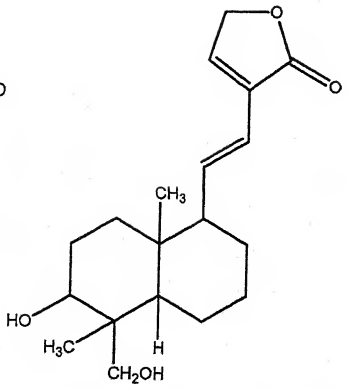
14-deoxyandrographolide



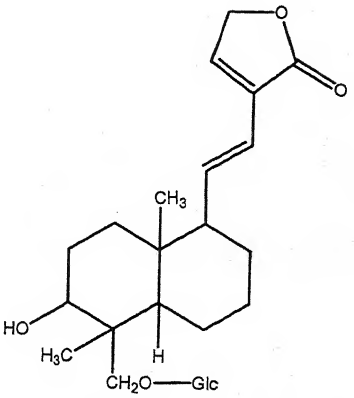
14-deoxyandrographiside



Andrographiside



14-deoxy-11,12-didehydro andrographolide



14-deoxy-11,12-didehydro andrographiside

Figure 9: Major chemical constituents of *Andrographis paniculata.*

The effect in common cold, fever, and inflammation was observed for all major andrographolides: deoxyandrographolide, andrographolide, neoandrographolide, and dehydroandrographolide. Dehydroandrographolide had the most pronounced effect, followed by neoandrographolide and andrographolide. The anti-inflammatory effect seemed to work by a mechanism that involved the adrenal glands. Further study confirmed that the anti-inflammatory action of dehydroandrographolide was due to its effect on increasing the synthesis and release of adrenocorticotrophic hormone (ACTH) of the pituitary gland of the brain. ACTH signals the adrenal gland to make cortisol, a natural anti-inflammatory (Yin and Guo, 1993).

Extracts of AP containing the four major active components were evaluated for antimalarial activity against *Plasmodium berghei*, one of the parasites that transmit malaria. The extract was found to produce considerably inhibition of multiplication of the parasites (Misra *et al.*, 1992). The effects of AP indicated the protective action due to reactivation of superoxide dismutase (SOD). AP extracts are also efffective in killing filaria (microscopic worms) that obstructs lymph channels in the body, leading to gross swelling termed elephantiasis. Since no toxic effects were apparent, researchers believed that the AP plant extract would be safe for humans and no plant has previously been shown to have antifilarial action. Screening of aqueous extracts of *Andrographis*, andrographolide and arabinogalactan proteins showed significant antibacterial and antifungal activities in comparison to some known antibiotics. The investigations revealed the biological value of the cumulative effects of AP and AD resulting in enhanced antimicrobial activity (Singh *et al.*, 2003).

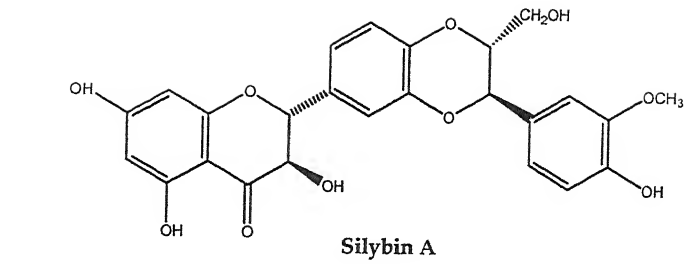
2.5.3 *Silybum marianum* (L.) Gaertn.

Silybum marianum, commonly known as "milk thistle" belongs to the family Asteraceae (or Compositae), is a herbaceous annual or biennial plant with a dense prickly flower head with purplish tubular flowers. Milk thistle is an

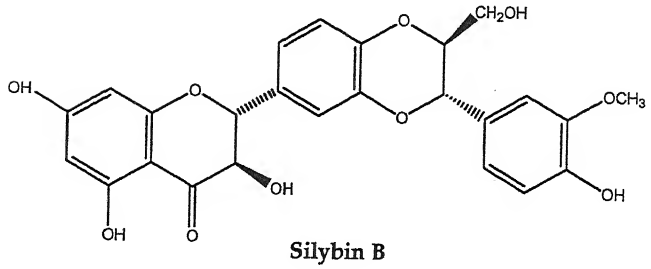
edible plant native to southern Europe, southern Russia, Asia Minor, and nothern Africa, and has been used for food in the countries surrounding the Mediterranean for a long time as well as a tonic herb for the liver. Virtually all parts of the plant have been used as food with no known toxicity. Its seeds and roots have been used for an assortment of medical purposes for thousands of years.

Silybum contained a wide range of molecules such as; flavonoids lignans (silymarin comprised of silybin, silydianin, and silychristin), essential oils, flavonoid (quercetin), tannins, and fixed Oil. The mixture of three biochemicals; silychristine, silydianin, and silybin in general, is called "silymarin." The biological mechanism of action is not well known but several theories exist: silymarin may control cell membrane permeability which means that silymarin may control what substances actually enter the interior of a cell; silymarin may inhibit chemical pathways leading to inflammatory biochemicals; silymarin may have free radical scavenging properties which means that it may absorb harmful reactive atoms that could damage other molecules; silymarin may increase protein production by liver cells; silymarin may stabilize mast cells (cells containing inflammatory granules); and, silymarin in higher doses increases the bile-flow (Fraschini *et al.*, 2002).

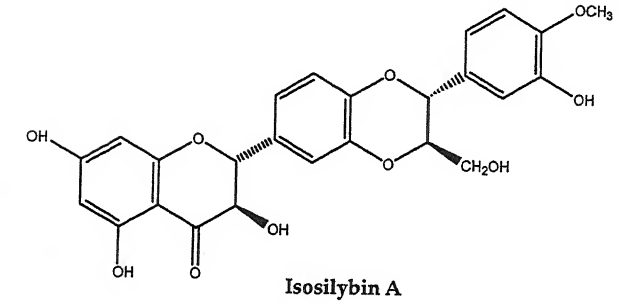
Some flavonoid glycosides are prepared synthetically or by biotransformations, usually for pharmaceutical purposes. Silybin is a flavonolignan used extensively as a potent hepatoprotectant and an antidote in mushroom poisoning. The only drawback of this compound is low water solubility; therefore its glycosylation was attempted by biological and chemical methods. Silymarin prevents uptake of the poison into the cells of the liver and thus, prevent the lethal liver damage associated with this type of mushroom poisoning Biotransformation led to the formation of silybin 7-β-glucoside and chemical glycosylations gave silybin glycosides at C-23 (β-glucoside, β-galactoside, β-maltoside and β-lactoside) (Kren *et al.*, 1998).



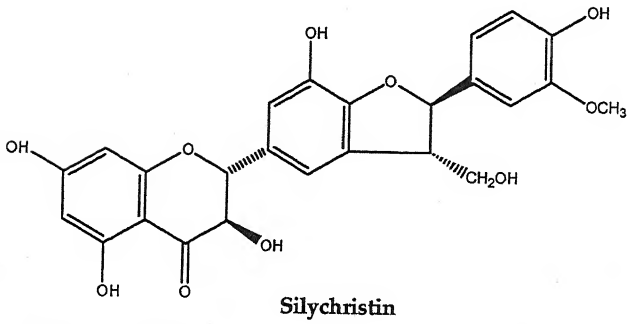
Silybin A



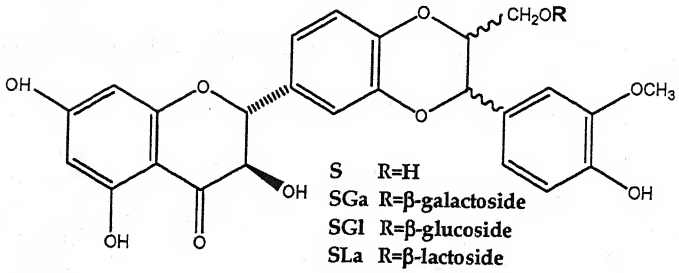
Silybin B



Isosilybin A



Silychristin



S R=H
SGa R=β-galactoside
SGl R=β-glucoside
SLa R=β-lactoside
SMa R=β-maltoside

Figure 10: Structure of major biomoleclues from *Silybum marianum*.

Solubility of the silybin glycosides was improved considerably compared to the aglycone. Biological tests showed that the silybin glycosides have a considerably higher radical scavenging activity. Silybin monoglycosides have also much better hepatoprotective activity than the aglycon in the tests measuring the release of lactate dehydrogenase from hepatocytes intoxicated with *t*-butylhydroperoxide. Similar effect was observed also in the tests with membrane lipoperoxidation of the mitochondrial membranes where silybin glycosides, mainly β-glucoside and β-galactoside, were found to be better antilipoperoxidants. Cytotoxicity of higher concentrations of silybin towards hepatocytes can be lowered also by its glycosylation - in this case especially β-maltoside and β-lactoside displayed substantially lower toxicity at high concentrations. Similarily, silybin β-galactoside was also found to have better hepatoprotective activities in vivo presumably due to β-galactosyl residue causing higher affinity towards hepatocytes (Kren *et al.*, 1998).

Silymarin is known to protect the liver by altering and strengthening the structure of outer cell membranes of hepatocytes (liver cells), preventing toxins from entering the liver cells, and by stimulating the regenerative ability of the liver and the formation of new hepatocytes through the activation of an enzyme nucleolar polymerase A, which leads to the increase in ribosomal protein synthesis and cell division (Flora *et al.*, 1998). Silymarin, as an anti-oxidant, may also reduce damages to liver cells caused by chronic use of certain prescription drugs. In some studies, *Silybum* is concerned to hypoglycemia, the patients treated with silymarin did not have any increase in the number of mild or severe hypoglycemic episodes, suggesting that silymarin stabilized as well as lowered glucose levels. In addition, SGOT and SGPT values declined significantly in the patients taking silymarin, confirming that liver function improved. There was also a decrease in blood levels of malondialdehyde, a marker of free radical damage, approaching that of healthy subjects (Flora *et al.*, 1998; Gazak *et al.*, 2004). Application of silybin/silymarin as a chemoprotective and anti cancer

agent could be considered in the first view for its antiradical potential and consequently for its cytoprotective activity. Owing to its chemopreventive effect silybin/silymarin inhibits carcinogenic action of many chemicals and significantly decreased the incidency of urinary bladder neoplasms and preneoplastic lesion in the initiation and post-initiation phase of the induction by N-butyl-N-(4-hydroxybutyl) nitrosamine. This compound also significantly limited azoxymethane-induced colon carcinogenesis in rats. Silymarin inhibited skin carcinogenesis induced by benzoyl peroxide or 12-O-tetradecanoylphorbol-13-acetate. Silybin or silymarin may be useful in treatment and prevention of some neurodegenerative and neurotoxic processes, partly due to its antioxidative activity. An extract from *Silybum marianum* seeds was tested on the differentiation and survival of cultured neural cells (rat PC-12 pheochromocytoma cell line). The extract enhanced the differentiation of PC-12 cells and prevented apoptosis following nerve growth factor (NGF) withdrawal. Moreover, the extract protected primary hippocampal neurons against oxidative stress-induced apoptosis (Kren and Walterova, 2005).

Silybin also interacts with other drug transporters, e.g., with multidrug resistance-associated protein 1 (MRP1). Influence of silymarin and other flavonoids was tested in human pancreatic adenocarcinoma cell line (Panc-1) on the transport of daunomycin and vinblastin. It was found that silymarin significantly increases accumulation of both drugs in the cells indicating the inhibition of MRP1. It seems that GSH regeneration is involved in this process because in the other study with flavonoids stimulation of GSH co-transport, ATPase and drug resistance-conferring properties of MPR1 were found to be modulated (Leslie *et al.*, 2001). Soluble derivatives of the hepatoprotective flavonolignan silybin, namely silybin galactoside, glucoside, lactoside and maltoside were investigated for their radical scavenging and antilipoperoxidation properties. Cyclic voltammetry results showed that glycosides are weaker electron donors than silybin, although it was of interest

that they were found to be more potent scavengers of the 1,1-diphenyl-2-picrylhydrazyl and the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)-derived radicals. The glycosides were more efficient than silybin in preventing tert-butylhydroperoxide-induced lipoperoxidation of rat liver mitochondrial membranes. Furthermore, glycosides were significantly more cytoprotective than silybin in tert-butylhydroperoxide-damaged rat erythrocytes and primary hepatocyte cultures. Glycosylation of silybin substantially reduced its toxic effects in primary cultured hepatocytes observed during prolonged incubation. These results suggest that silybin glycosides are suitable soluble derivatives of silybin for experimental studies and may have therapeutic potential (Kosina *et al.*, 2002).

2.6. Fucosyltransferase

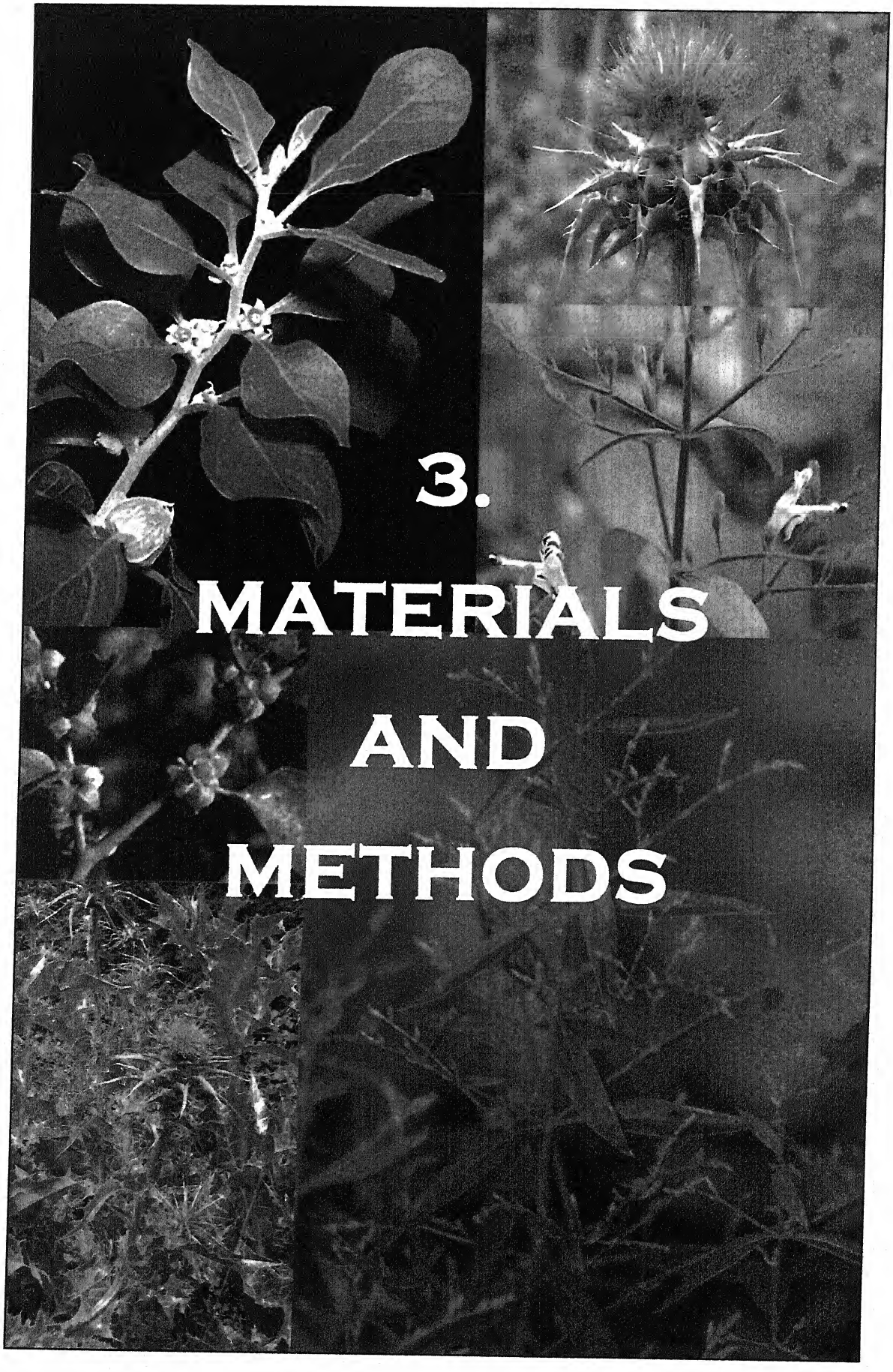
An off-shoot of this study on *Silybum marianum* proteomic analyses has resulted in the identification of a putative fucosyltransferase. Since as such there is little investigation on fucosyltransferases from plants, the relevant literature on them has been presented here holistically on plant and non-plant sources like *Arabidopsis thaliana, Oryza sativa, Populus* (*Populus tremula* x *Populus alba*), and *Homo sapiens* (Perrin *et al.*, 1999; Sasaki *et al.*, 2002; Costa *et al.*, 2007; Kelly *et al.*, 1995)

Fucosyltransferase catalyzes transfer of L-fucose from a GDP-fucose (Guanosine diphosphate-fucose) donor to an acceptor leading to biosynthesis of fucosides. The acceptor substrate can be another sugar such as the transfer of a fucose to a core GlcNAc (*N*-acetylglucosamine) sugar as in the case of *N*-linked glycosylation, or a protein, as in the case of *O*-linked glycosylation produced by *O*-fucosyltransferase. Fucosyltransferase activity may be a risk factor for urinary tract infection. GDP-fucose protein *O*-fucosyltransferase 1 (PoFUT1) is an enzyme responsible for adding fucose sugars in *O* linkage to serine or threonine residues between the second and third conserved cysteines in EGF-like repeats on the

Notch protein. The protein is an inverting glycosyltransferase, which means that the enzyme uses GDP-β-L-fucose as a donor substrate and transfers the fucose in O linkage to the protein producing fucose-*α*-*O*-serine/threonine. Almost all glycosyltransferases reside in the Golgi apparatus. However, PoFUT1 as well as the related enzyme PoFUT2 have recently been shown to reside in the endoplasmic reticulum (Costache *et al.*, 1997).

Plant cell walls play a crucial role in development, signal transduction, and disease resistance. They are made of cellulose and matrix polysaccharides such as hemicelluloses and pectins. Xyloglucan, the principal hemicellulose of dicotyledonous plants, has a terminal fucosyl residue that may affect the extensibility of the cell wall and thus influence plant growth and morphology. A key component in the association between cellulose and xyloglucan is the presence of the L-fucose-containing trisaccharide side-chain. Computer modeling of xyloglucan structure predicts that fucose containing xyloglucans adopt spatial conformations more favorable for cellulose binding than nonfucosylated xyloglucan (Levy *et al.*, 1991). Fucosylated xyloglucans bind cellulose *in vitro* at a 2-fold higher rate than do nonfucosylated xyloglucans (Levy *et al.*, 1997); however, xyloglucan fucosylation is not absolutely required for the formation of cellulose–xyloglucan networks (Whitney *et al.*, 1995). Fucose-containing xyloglucan is also thought to play a role in the regulation of plant growth. Several studies have shown that xyloglucan-derived oligosaccharides, called oligosaccharins, act as inhibitors of auxin-stimulated elongation of pea epicotyls. The biological activity of these oligosaccharins depends on the presence of terminal L-fucose (Cote and Hahn, 1994).

There are various fucosyltransferases in mammals, the vast majority of which are located in the Golgi apparatus however *O*-fucosyltransferases have recently been shown to be localized to ER also. Compared to animals, very few fucosyltransferases are known and far little characterized from plant world.



3. MATERIALS AND METHODS

The wide metabolic application of β-glucosidase has prompted to study the physico-kinetic characteristics of β-glucosidases in selected medicinal plants: *Withania somnifera, Andrographis paniculata* and *Silybum marianum*. Since the majority of the glycosides have glucose as the glycan moiety, it is proposed to carryout the isolation, purification and characterization of β-glucosidase and comprehend a comparative account of their catalytic kinetics. The methodologies recruited for carrying out (i) profiling of β-glucosidase level in different plant parts in the selected medicinal plants, (ii) isolation and purification of β-glucosidases from selected medicinal plants, (iii) elucidation of comparative physico–kinetic characteristics of catalysis by the selected enzymes; substrate specificity and regulatory properties of the purified enzymes, (iv) molecular characterization of purified enzyme with respect to limited amino acid sequence or peptide mapping in selected case(s), (v) exploration of catalytic feasibility to carry out transglycosylation under designed conditions are presented here.

Since some of the technical elements were specific to plant resource (*Withania somnifera, Andrographis paniculata* and *Silybum marinum*) of the β-glucosidase, the materials and methods for their investigation have been presented independently as plant specific β-glucosidase sections.

3.1. Purification and physico-kinetic characterization of *Withania somnifera* leaf β-glucosidase

3.1.1. Plant Material

Withania somnifera accession RSS-1 plants were raised at the experimental farm of Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow (India) following standard agronomic practices. Young leaves were harvested for isolation of the enzyme.

3.1.2. Chemicals

All biochemicals and reagents like chromogenic substrates (*p*-nitrophenyl β-D-glucopyranoside and other substrate analogues), sodium gluconolactone, buffers components etc. were purchased from Sigma Chemicals Co. USA. Electrophoresis chemicals and reagents were procured from Sigma or Bio-Rad. Chromatographic materials like Sephadex G-25, Sephadex G-75, Q-Sepharose, S-Sepharose and octyl-Sepharose CL-4B were from M/s Amersham Pharmacia Biotech. All other chemicals were of highest purity from E. Merck, Sisco Research Laboratories (Mumbai, India) or Spectrochem (Mumbai, India). Other specifically used chemicals/biochemicals, tools and techniques were find their description at required place. All the buffers and reagents were prepared in triple distilled water made in Quartz-condenser distillation unit (M/s Bhanu Scientific Co., India).

3.1.3. Optimization of enzyme isolation and assay

To discern optimal extraction of β-glucosidase (specific activity) from *Withania somnifera* leaf, the enzyme was isolated using extraction buffers of different pH- 100 mM citrate-phosphate pH 2.5, 3.0, and 100 mM potassium-phosphate buffer pH 6.0. All the extraction buffers contained 10 mM β-mercaptoethanol, 5 mM thiourea and 2 mM EDTA. The homogenate was centrifuged at 10,000 x g for 30 min at 4 °C. The enzyme activity was assayed in the supernatant and the total protein content was estimated to compute specific activity. *A priori,* assays were carried out to optimize conditions for linearity with respect to time and enzyme concentration.

3.1.4. Enzyme extraction and purification

All the steps of enzyme extraction and purification were carried out 0-4 °C unless specified otherwise. Leaf tissue (1.10 Kg) was immediately surface rinsed

with distilled water, de-moistened with a tissue paper and powdered with liquid nitrogen in a pestle-mortar.

The powder was homogenized in one volume (w/v) of 100 mM potassium-phosphate buffer pH 2.5 containing 10 mM β-mercaptoethanol, 5 mM thiourea and 2 mM EDTA. The homogenate was filtered through four layers of muslin cloth and the filtrate was centrifuged at 10,000 x g (30 min). The clear supernatant (1.51 L) was collected, assayed for enzyme activity and subjected to purification steps.

3.1.4.1. Ammonium sulfate fractional precipitation and desalting

The supernatant was subjected to differential solid ammonium sulfate precipitation and the protein fraction precipitating between 40-60% saturation was collected by centrifugation at 10,000 x g (10 min), dissolved in minimum volume of 10 mM potassium phosphate buffer pH 6.0 and desalted through a Sephadex G-25 (2.0 x 25 cm; void volume 15.0 ml; flow rate 1.0 ml min^{-1}) pre-equilibrated with 10 mM potassium-phosphate buffer pH 6.0 and eluted with the same buffer. Fractions (2.0 ml) with significant catalytic activity were pooled and preceded to further step of purification.

3.1.4.2. Cation exchange chromatography on S-Sepharose column

The enzyme pool of Sephadex G-25 column was subjected to cation-exchange chromatography using S-Sepharose column (1.0 x 10 cm; flow rate 0.75 ml min^{-1}) pre-equilibrated and eluted with 10 mM potassium-phosphate buffer pH 6.0 followed by elution with the buffer containing 1 M KCl. Fractions (2.0 ml) were collected and monitored for protein content (A_{280}) and enzyme activity. The fractions with enriched specific activity were pooled and subjected to hydrophobic interaction chromatography.

3.1.4.3. Hydrophobic interaction chromatography on octyl-Sepharose CL-4B column

The enzyme preparation of S-Sepharose column was added with ammonium sulfate to final concentration of 1.7 M and loaded onto an octyl-Sepharose CL-4B column (1.0 x 10 cm; flow rate 0.75 ml min^{-1}). The column was pre-equilibrated with 1.7 M ammonium sulfate in 10 mM potassium-phosphate buffer pH 6.0 and, post-sample load, was successively eluted with 10 mM potassium-phosphate buffer (pH 6.0) containing 1.7 M ammonium sulfate and containing 1.0 M ammonium sulfate. The eluted fractions (2.0 ml) were collected, screened for native protein at 280 nm and then enzyme activity was assayed. The high activity containing fractions were pooled and precipitated with ammonium sulfate (80% saturation) and the pellet was recovered after centrifugation at 10,000 x g for 10 min at 4 °C, desalted through Sephadex G-25 column (25 x 2.0 cm; bed volume 78.5 ml; void volume 15.0 ml; flow rate 1.0 ml/min) pre-equilibrated and eluted with 10 mM potassium-phosphate buffer pH 6.0.

3.1.4.4. Anion exchange chromatography on Q-Sepharose column

The desalted enzyme preparation from previous purification step was loaded onto an anion exchange (Q-Sepharose) column (1.0 x 10 cm; flow rate 0.75 ml min^{-1}) pre-equilibrated by 10 mM potassium-phosphate buffer pH 6.0. The column was sequentially eluted with step-wise salt gradient of 0.1 M and 0.2 M NaCl in the equilibration buffer. The fractions (2.0 ml) were monitored for protein and enzyme activity and active fractions eluted under 0.2 M NaCl were pooled and again subjected to cation-exchanger S-sepharose.

3.1.4.5. Cation exchange chromatography on S-Sepharose column

The enzyme pool of Q-Sepharose column was again subjected to cation exchange chromatography through S-Sepahrose column (1.0 x 1.0 cm; flow rate 0.75 ml min^{-1}) and eluted in similar fashion as eluted earlier on the same column.

The active fractions displaying single polypeptide band in SDS-PAGE were pooled and subjected to physico-kinetic characterization.

3.1.5. Enzyme assay

β-Glucosidase activity was assayed by the modified method of Stevens *et al.* (1993) using *p*-nitrophenyl-β-D-glucopyranoside. The assay mixture in a total volume of 200 μL contained 2.5 mM *p*-nitrophenyl-β-D-glucopyranoside, 100 mM citrate-phosphate buffer (pH 4.8) and the enzyme preparation. The reaction was started by the addition of enzyme and the reaction run at 40 °C for 10 min following which the reaction was stopped by the addition of 800 μL of 1.0 M sodium carbonate and the rate of formation of aglycone (*p*-nitrophenol) was monitored in terms of increase in absorbance at 405 nm in a UV-visible spectrophotometer (Perkin Elmer). The control, that contained all the assay mixture components but wherein the reaction was stopped at time zero, was run parallel to set the background absorbance to zero. One unit of enzyme activity was defined as that catalyzing formation of one μmole of *p*-nitrophenol formed per minute, under the defined conditions, computed using molar absorption coefficient (ε°) value of 18,350 $M^{-1}.cm^{-1}$ for *p*-nitrophenol (Estibalitz *et al.*, 2001).

3.1.6. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was run in a Mini-PROTEAN-II gel electrophoresis system (BioRad) using modified method of Doucet and Trifaro (1988). The discontinuous gel consisted of a 12% (acrylamide + bisacrylamide) resolving gel (5.5 cm) and 4.0% stacking gel (0.5 cm) containing 0.4% SDS, 5% glycerol and 200 mM Tris 100 mM glycine pH 9.0, and 4.5% stacking gel (0.5 cm) containing 0.4% SDS, 4 mM ethylenediamine N,N,N',N'-tetraacetic acid (EDTA), 5% glycerol and 70 mM Tris-HCl buffer pH 6.8. The gels were polymerized with the addition of 0.15% each of TEMED and ammonium per sulfate. The SDS-samples were prepared by

heated them in boiling water bath (3 min) after the addition of equal volume of SDS-sample buffer (200 mM Tris-HCl pH 6.8 containing 10% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.025% bromophenol blue and urea). The samples (1 μg protein each) were loaded and upper chamber electrode buffer was 20 mM Tris 30 mM glycine pH 8.3 with 0.02% SDS. The gel was run at room temperature on 120 v for stacking gel and 80 v for resolving gel. Bromophenol blue was used as tracker dye. At the end of the run, the gel was subjected to silver staining. A mixture of standard MW protein was co-electrophoresed.

Silver staining: After the SDS-PAGE was run the gel was stained with modified silver staining method of Blum *et al.* (1987). Silver staining was performed using highly précised chemicals for electrophoresis and avoiding any hand or other contaminants exposure to gel. After running the gel was scooped from gel plates and merged in fixing reagent, 40% methanol 10% acetic acid in water for 2 hour. Thereafter the gel was placed in reconditioning solvent buffer system containing 10% methanol 5% acetic acid in water for 1 hour. Then gel was placed in a solution of 3.4 mM potassium dichromate in 3.2 mM nitric acid in water for 5 min. After dichromate exposure gel becomes yellow in color which was subsequently removed by washing with water for several times until yellow color of gel goes off. Then placed the gel in 24 mM silver nitrate solution for 20 min covered with black foil to avoid light exposure as well as gel was kept rocking on gel rocker. The all above mentioned procedures were put on extreme time dependent manner to avoid over/under staining of gel. Then washed the gel twice with water and further step of band development was performed under strict vigilance to better resolution of protein bands on gel. An alkaline formaldehyde solution containing 0.28 M sodium carbonate and 3.7% formaldehyde was applied on gel, gel was kept rocking and the solution was discarded after its color changes to blackish. This process was repeated for three times with fresh alkaline solution until clear and specific protein bands were

visualized and the background of gel become slightly yellow in color. After band visualization the gel was merged in 3% acetic acid solution for 10 min to stop further image development and finally gel was placed in 10% ethanol solution for storage purpose.

3.1.7. Preparative isoelectric focusing

The preparatory ioselectric focusing of *Withania* β-glucosidase was performed on the Rotofor Cell (BioRad) using the ampholyte of pH range 3-10. The focusing was run for 5 hours at 15 w constant power and temperature of the whole unit was maintained to 4 °C with continuous chilled water supply in cooling fingers of the unit. After the isoelectric zones were separated inside the focusing chamber zones of different *p*I were harvested separately under vacuum and analyzed for pH and enzyme activity in subsequent fractions. From the analyses based on preparative isoelectric focusing the isoelectric point (*p*I) for *Withania somnifera* β-glucosidase was recorded.

3.1.8. Protein estimation

Protein estimation was done by the method of Lowry *et al.* (1951) using bovine serum albumin as reference standard.

3.1.9. Native molecular weight and subunit composition

Native and subunit molecular weight of the purified enzyme were deciphered from the Sephadex G-75 (1.8 x 40 cm) column chromatography and SDS-PAGE subsequently. The void volume of the Sephadex G-75 column was determined by eluting blue dextran R-200 dye (0.5 ml of 2.0 mg ml^{-1}) in 10 mM phosphate buffer pH 6.0 at a flow rate of 1.0 ml min^{-1}. The column was calibrated with standard native protein molecular weight markers (M/s Amersham Pharmacia Biotech.). One mg each of bovine serum albumin (67,000), ovalbumin (43,000), and chymotrypsin (25,000) were loaded and eluted with 10 mM

phosphate buffer pH 6.0 and a calibration plot was drawn between the $\log_{10}$ molecular weight *versus* elution volume to discern the molecular weight.

On SDS-PAGE standard protein molecular weight markers (M/s Banglore Genie) were co-electrophoresed which contained phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,100), and lysozyme (14,300). The subunit molecular weight of *Withania* β-glucosidase was deciphered from the plot drawn between $\log_{10}$ molecular weight *versus* migration distance.

3.1.10. pH optima and pH stability

To estimate the pH optima of the enzyme, assay mixture was developed at different assay buffer 100 mM citrate-phosphate pH ranging from 3.0 to 9.0. Rest of the assay conditions were kept constant with proper controls made as per change required and the activity was recorded. The enzyme stability against varying pH buffers was observed to find its best suitable media. A fraction of enzyme pool was passed though Sephadex G-25 column and desalted against water so that pH remain neutral. A 100 µL enzyme was mixed with 100 µL of 100 mM citrate-phosphate pH 3.0, 4.0, 5.0, 6.0, 7.0 and 100 mM Tris-buffer pH 8.0, 9.0 to a final concentration of 50 mM and enzyme activity was assayed after every 30 min, 1 h, 2 h, and 4 h at 40 °C and 4.8 pH.

3.1.11. Thermostability and thermotropic behaviour

The optimal activity of enzyme for temperature was estimated by assaying activity of standard reaction mixture at different incubation temperatures ranging from 4-80 °C. In a similar fashion, the enzyme stability against temperature was observed by keeping enzyme at different storage temperatures ranging from 4-70 °C and enzyme activity was assayed from such enzyme aliquots in standard assay mixture at 40 °C and pH 4.8 after every 30 min, 1 h, 2 h, and 4 hour.

3.1.12. Substrate saturation kinetics

The catalytic functionality of the enzyme was monitored by measuring enzyme activity at different substrate concentrations. The enzyme reaction mixture contained 0.05 to 0.40 mM substrate (*p*-nitrophenyl-β-D-glucopyranoside) in 100 mM citrate-phosphate buffer pH 4.8 at 40 °C for 5 min. The kinetics plots for enzyme catalysis were drawn and K_m and V_{max} were determined, the Michaelis-Menten plot was drawn using GraphPad™ scientific software in non-linear regression mode.

3.1.13. Substrate specificity

To decipher the substrate specificity of enzyme different substrate analogues were tested for activity. The substrates used *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *o*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-β-D-lactopyranoside and 6-bromo-2-naphthyl β-D-glucopyranoside were 2.5 mM in final reaction mixture of 100 mM citrate-phosphate pH 4.8 at 40 °C for 10 min.

3.1.14. Inhibition kinetics

D(+)-Glucono-1,5-lactone was used to assess its effects on *Withania* β-glucosidase in the range of 0.01-2.0 mM concentration. One ml assay mixture contained 100 mM citrate-phosphate buffer pH 4.8, 10 μL enzyme extracts and 2.5 mM substrate (*p*NPG) along with varying concentrations of inhibitor at optimal reaction conditions. The enzyme activity in presence of inhibitor was recorded and the inhibition kinetics was elaborated.

3.1.15 Effect of cations, chelating agents and thiol directed reagents

In order to screen the effectors of β-glucosidase several metal ions, chelating agents and several other compounds were tested for their effect against enzyme activity. The metals ions were mono-, di-, and tri-valent cations like Li^+, K^+, Na^+, $NH4^+$, Ag^+, Hg^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, and Fe^{3+}; chelating agents like ethylenediamine N,N,N′,N′-tetraacetic acid (EDTA) and ethyleneglycol N,N,N′,N′-tetraacetic acid (EGTA); and other compounds like iodoacetate, iodoacetamide, phenyl methyl sulphonyl fluoride (PMSF), *p*-chloro-mercuric benzoic acid (PCMB), and benzidine. The reaction mixture contained each effector in 1.0, 2.5, and 5.0 mM concentrations with 10 µL enzyme in 100 mM citrate-phosphate buffer pH 4.8 at optimal assay conditions. The reaction mixture was incubated for 10 min and before adding substrate a pre-incubation of 10 min was given to enzyme-effector state.

3.1.16. Effect of organic solvents

Four organic solvents ethanol, methanol, acetone and dimethyl sulphoxide (DMSO) were tested for activity against enzyme at varying concentrations. One ml assay mixture contained 100 mM citrate-phosphate buffer pH 4.8, 5 µL enzyme extracts and 2.5 mM substrate (*p*NPG) with different organic solvents under optimal reaction conditions. Before adding substrate a pre-incubation of 10 min was given to enzyme-solvent state and then activity was recorded in after 10 min of incubation.

3.1.17. Effect of glucose

Effect of glucose on enzyme activity was estimated by adding glucose in varying concentration (0.1, 0.2, 0.4, 0.5, 1.0, 2.0, 4.0, 10, 20, 40 mM) in standard reaction mixture of 1.0 ml containing 2.5 mM substrate (*p*NPG) in 100 mM citrate-phosphate buffer pH 4.8. The assay conditions were kept standard and a pre-incubation of 10 min was given to enzyme-glucose state before adding substrate.

3.1.18. Transglycosylation

To assess the transglycosylating catalytic feasibility of the *Withania* β-glucosidase under lower aqueous micromilieu generated through DMSO (10%) the transglycosylation assay mixture contained different aglycone acceptors like methanol, ethanol, propanol, butanol, pentanol, hexanol, hepatanol, octanol, decanol, geraniol, linalool, menthol, and withaferin A. A preliminary screening of effect of the aglycone molecules was estimated in standard assay mixture containing 20 mM aglycone with 2.5 mM susbtrate (*p*NPG) in 100 mM citrate-phosphate buffer pH 4.8. The enzyme activity in presence of aglycone molecule was recorded spectrometrically with proper controls.

The transglycosylation reaction was designed in two sets depending on glycosyl donor; cellobiose and *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) were used as glucosyl donors. Cellobiose-dependent transglycosylation reaction mixture (1.0 ml) contained 50 mM each of 1-phenylethanol, 2-phenylethanol, geraniol, linalool, menthol and withaferin A as acceptors (aglycone); cellobiose 2 mM with 10 µl of enzyme in buffering of 100 mM citrate-phosphate pH 4.8. The reaction mixture was incubated at 30 °C for 18 h After incubation the reaction mixture was fractionated with 1.0 ml ethyl acetate for 4 times and ethyl acetate part was evaporated to dryness and dissolved in 200 µl of methanol for thin layer chromatography.

*p*NPG-dependent reaction mixture (0.5 ml) contained 20 mM of each aglycone acceptors (geraniol, linalool, menthol, butanol and withaferin A), 40 mM *p*-nitrophenyl-β-D-glucopyranoside with 10 µl of enzyme in buffering of 100 mM citrate-phosphate pH 4.8. The reaction mixture was incubated at 30 °C for 18 h After incubation the reaction mixture was fractionated with 1.0 ml ethyl acetate for 4 times and ethyl acetate part was evaporated to dryness and dissolved in 200 µl of methanol for thin layer chromatographic analysis.

Thin layer chromatography of transglycosylation product was run on pre-coated silica gel TLC plates (E. Merck) in a solvent system of chloroform : ethyl

acetate : methanol (70 : 6 : 10 v/v/v). TLC plates were sprayed with 5% sulfuric acid in ethanol and then 1% vanillin in ethanol and heated at 110 °C for 10 min. Development of colored spots were observed and R_f was calculated for comparative analysis.

3.2. Purification and physico-kinetic characterization of *Andrographis paniculata* leaf β-glucosidase

3.2.1. Plant material

Andrographis paniculata plants were raised at the experimental farm of Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow following standard agronomic practices. Young (~50% expanded) leaves were sampled for the enzymatic work.

3.2.2. Chemicals

All chemicals used were as described under the head **3.1.2** for *W. somnifera* enzyme.

3.2.3. Enzyme isolation optimization

To establish the suitable extraction media for high specific activity of *Andrographis* β-glucosidase, the enzyme was isolated in different pH buffers 100 mM citrate-phosphate pH 2.0, 2.5, 3.0, 3.5, 4.5 and 100 mM potassium-phosphate buffer pH 6.0, including 10 mM β-mercaptoethanol, 5 mM thiourea and 2 mM EDTA. The homogenate was centrifuged at 10,000 x g for 30 min at 4 ºC and supernatant was used to assay enzyme activity and total protein estimation for specific activity.

3.2.4. Enzyme extraction and purification

Leaf tissue (60 g) was, immediately after harvest, powdered in liquid nitrogen in an all glass pestle and mortar and extracted with two-volumes (w/v) of 100 mM citrate-phosphate buffer (pH 3.0) containing 10.0 mM 2-mercaptoethanol, 5.0 mM thiourea and 2.0 mM EDTA in the presence of 1% (w/v) insoluble PVPP (Polyclar AT). The homogenate was centrifuged at 10,000 x g for 30 min at 4 °C, clear supernatant (110 ml) was collected, assayed for β-glucosidase activity and subjected to purification. All the steps of enzyme extraction and purification were carried out at 0-4 °C unless specified otherwise.

3.2.4.1. Ammonium sulfate fractional precipitation and size exclusion chromatography through Sephadex G-75 column

The supernatant was subjected to differential solid ammonium sulfate precipitation and the protein fraction precipitating between 0-40% ammonium sulfate saturation was collected by centrifugation (10,000 x g, 10 min), dissolved in 10 mM potassium-phosphate buffer pH 6.0 (8.0 ml). The $(NH_4)_2SO_4$ preparation was loaded on a Sephadex G-75 column (1.8 x 50 cm; void volume 40 ml; flow rate 1.0 ml min^{-1}) pre-equilibrated with 10 mM potassium-phosphate (pH 6.0) buffer and eluted with the same buffer. Fractions (2.0 ml) were collected, screened for protein content (A_{280}) as well as β-glucosidase activity and catalytically active fractions were pooled for anion exchange chromatography.

3.2.4.2. Anion exchange chromatography

Anion exchange chromatography was performed on a Q-Sepharose column (1.5 x 10 cm; flow rate 0.75 ml min^{-1}) pre-equilibrated with 10 mM potassium-phosphate buffer (pH 6.0), sequentially eluted with buffer followed by the buffer containing 0.1 M NaCl and 2.0 ml fractions were collected, monitored for protein (A_{280}) and the enzyme activity. The fractions eluted under salt that had

significant catalytic activity were pooled, added with $(NH_4)_2SO_4$ to a final concentration of 1 M and subjected to hydrophobic interaction chromatography.

3.2.4.3. Hydrophobic interaction chromatography (HIC)

HIC was carried out on an octyl-Sepharose column (1.5 x 8 cm; flow rate 0.75 ml min^{-1}) pre-equilibrated with 10 mM potassium-phosphate buffer (pH 6.0) containing 1 M $(NH_4)_2SO_4$. The enzyme preparation containing 1 M $(NH_4)_2SO_4$ was loaded and the column was with the buffer containing step-wise decreeing (1 M to 0 M) concentration of $(NH_4)_2SO_4$ and fractions collected were monitored for protein (A_{280}) and enzyme activity. The enzyme activity was observed in the fractions when the column was finally eluted with distilled water. The fraction that had highest specific activity was subjected to SDS-PAGE analysis and used for physico-kinetic characterization.

3.2.5. Enzyme assay

β-Glucosidase activity was assayed by the modified method of Stevens *et al.* (1993) (as detailed in section **3.1.5.**) using *p*-nitrophenyl β-D-glucopyranoside in assay buffer 100 mM citrate-phosphate pH 5.5 and incubation temperature 55 °C.

3.2.6. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was run in a Mini-PROTEAN-II gel electrophoresis system (BioRad) using modified method of Doucet and Trifaro (1988) (as detailed in section **3.1.6.**). The samples loaded were 5 μg per well each except final purified preparation which was 0.035 μg.

3.2.7. Non-denaturing polyacrylamide gel electrophoresis (native-PAGE)

For *In situ* localization of β-glucosidase native-PAGE was run in BioRad Mini PROTEAN-II gel electrophoresis system with modified method of Doucet

and Trifaro (1988). A 5.5 cm non-denaturing separating (running) gel of 7% polyacrylamide and bisacrylamide was prepared in 375 mM Tris-HCl buffer pH 8.8 with 5% glycerol. A 0.5 cm stacking gel with 1.0 cm wells was also prepared over separating gel which contained 50 mM Tris-HCl buffer pH 8.5 with 5% glycerol. The 25 μL loaded sample was premixed with 10% glycerol and the gel was run at 120 v for 15min (until the sample was in wells) and then 80 v for 2.5 hour (until the tracking dye-bromo phenol blue was eluted at edge of gel) in electrode buffer of composition 25 mM Tris 186 mM glycine pH 8.3 at 4 °C.

Activity staining: After the native-PAGE was run the activity based staining of gel was carried out by the method of Esen (1992) and Fieldes and Gerhardt (1994). Briefly, the native PAGE gel was first washed with 50 mM citrate phosphate buffer pH 5.5 for 5 min and transferred to staining system containing 1.3 mM 6-bromo-2-naphthyl β-D-glucopyranoside and 1.9 mM fast blue BB salt (coupling dye) in 100 mM citrate phosphate buffer pH 5.5. Gel was kept in covered glass tray in dark at 37 °C for 18 hour, then placed the gel in fixing solution of acetic acid : methanol : water (1:1:5 v/v/v) for 24 hour.

3.2.8. Preparative isoelectric focusing

The preparative ioselectric focusing of *Andrographis* β-glucosidase was performed on the Rotofor Cell (BioRad) using pH range 3-10 ampholyte. The isoelectric focusing was run for 3.5 h at constant power (15 w). The temperature of the resolving chamber was maintained to 4 °C by water circulation through cooling fingers of unit using a refrigerated circulatory waterbath. At the end of focusing, the contents from different zones of the chamber were collected by vacuum pump-aided evacuation and each fraction analyzed for pH and enzyme activity and the isoelectric point (*p*I) for *Andrographis paniculata* β-glucosidase was discerned from the data sets.

3.2.9. Protein estimation

Protein estimation was done by the method of Lowry *et al.* (1951) using bovine serum albumin as reference standard.

3.2.10. Native molecular weight and subunit composition

Native and subunit molecular weight of the purified enzyme were deciphered from the Sephadex G-75 (50 x 0.9 cm; bed volume 127.17 ml; void volume 40 ml; flow rate 1.0 ml/min) column chromatography and SDS-PAGE subsequently. The Sephadex G-75 column was calibrated with standard native protein molecular weight markers (M/s Amersham Pharmacia Biotech.). One mg each of bovine serum albumin (67,000), ovalbumin (43,000) and chymotrypsin (25,000) were loaded and eluted with 10 mM phosphate buffer pH 6.0 and a calibration plot was drawn between the $\log_{10}$ molecular weight *versus* average elution volume (K_{av}). The SDS-PAGE for purification samples was co-electrophoresed with standard protein molecular weight markers; phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (40,000), carbonic anhydrase (26,600), soybean trypsin inhibitor (21,500), and lysozyme (14,400) (M/s Biogene, USA). The subunit molecular weight of *Andrographis* β-glucosidase was deciphered from the plot drawn between $\log_{10}$ molecular weight *versus* migration distance.

3.2.11. pH optima and pH stability

The pH optimum of the enzyme was obtained from the assay mixtures developed with different assay buffer 100 mM citrate-phosphate pH ranging from 3.0 to 10.0. Rest of the assay conditions were kept constant with proper controls and the enzyme activity was recorded. To find the pH stability of the enzyme, a fraction (1.0 ml) of enzyme preparation was passed though Sephadex G-25 column and desalted against water so that pH remain neutral. A 100 μL enzyme was mixed with 100 μL of 100 mM citrate-phosphate pH 3.0, 4.0, 5.0, 6.0,

7.0 and 100 mM Tris-buffer pH 8.0, 9.0, 10.0 to a final concentration of 50 mM and enzyme activity was assayed after every 30 min, 1 h, 2 h, and 4 h at 55 °C and 5.5 pH.

3.2.12. Thermostability and thermotropic behaviour

The optimal activity of enzyme for temperature was estimated by assaying activity of standard reaction mixture at different incubation temperatures ranging from 10-80 °C with 10 °C differences. The enzyme stability against temperature was observed by keeping enzyme at different storage temperatures ranging from 4-80 °C and enzyme activity was assayed from such enzyme aliquots in standard assay mixture after every 30 min, 1 h, 2 h, and 4 h at 55 °C and 5.5 pH.

3.2.13. Substrate saturation kinetics

The catalytic functionality of the enzyme was monitored by measuring enzyme activity at different substrate concentrations. The reaction mixture contained 0.02, 0.06, 0.10, 0.14, 0.18, 0.22, 0.26, 0.30, 0.34, 0.38, 0.42, 0.46, and 0.50 mM substrate (*p*-nitrophenyl-β-D-glucopyranoside) at optimal reaction conditions for 5 min. The K_m and V_{max} functions were determined from the kinetics plots drawn for enzyme catalysis on GraphPad™ scientific software in non-linear regression mode.

3.2.14. Substrate specificity

To interpret the substrate specificity of enzyme different substrate analogues were tested for activity. The substrates used *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *o*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-

fucopyranoside, 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-Glu) and 6-bromo-2-naphthyl β-D-glucopyranoside were 2.5 mM in final reaction mixture at optimal reaction conditions for 10 min.

3.2.15. Inhibition kinetics

The inhibition pattern of D-gluconic acid lactone was projected for *Andrographis* β-glucosidase in the range of 0.5 to 50 mM concentration. The varying inhibitor concentration was applied in 1.0 ml assay mixture containing 2.5 mM substrate (*p*NPG) and 10 μL enzyme preparation in 100 mM citrate-phosphate buffer pH 5.5 at 55 °C for 10 min. The enzyme activity in presence of inhibitor was recorded and the inhibition kinetics was convoluted.

3.2.16. Effect of glucose

The enzyme activity in presence of glucose was estimated by varying glucose concentration (0.5-40 mM) in standard reaction mixture of 1.0 ml containing 2.5 mM substrate (*p*NPG) in 100 mM citrate-phosphate buffer pH 5.5. A pre-incubation of 10 min was given to enzyme-glucose state before adding substrate to assay mixture and the enzyme activity was estimated after 10 min incubation at 55 °C.

3.2.17. Effect of cations

Effect of several metal ions, and other compounds were tested for their effect against β-glucosidase activity. Among metals ions mon-, di-, and tri-valent cations like Li^{+}, K^{+}, Na^{+}, Ag^{+}, Hg^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, and Fe^{3+} were screened. The reaction mixture contained each effector in 1.0 and 10 mM concentrations with 10 μL enzyme in 100 mM citrate-phosphate buffer pH 5.5 at 55 °C. The reaction mixture was incubated for 10 min and before adding substrate a pre-incubation of 10 min was given to enzyme-effector state.

3.2.18. Effect of organic solvents

Water miscible organic solvents like methanol, ethanol, acetone and dimethyl sulphoxide (DMSO) were tested for effect on enzyme activity at varying concentrations. In 1.0 ml assay mixture solvents concentration varied 2-60% with 10 μL enzyme preparation and 2.5 mM substrate (*p*NPG) in 100 mM citrate-phosphate buffer pH 5.5. Before adding substrate a pre-incubation of 10 min was given to enzyme-solvent state and then activity was recorded in after 10 min of incubation at 55 °C.

3.2.19. Transglycosylation

The transglycosylation activity of *Andrographis* β-glucosidase was assessed by the method described in previous section **3.1.18.** with only difference in the assay buffer, 100 mM citrate-phosphate pH 5.5.

3.3. Purification and physico-kinetic characterization of *Silybum marianum* petal β-glucosidase

3.3.1. Plant material

Silybum marianum plants were raised at the experimental farm of Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow following standard agronomic practices. Complete flowers (thistle) were harvested and petals were sampled for the enzymatic work.

3.3.2. Chemicals

All chemicals used were as described in head **3.1.2.** except few specifically used which are described at appropriate place.

3.3.3. Enzyme isolation optimization

Silybum β-glucosidase petals (0.5 gm) was extracted in double volume (w/v) different pH extraction buffers 100 mM citrate-phosphate pH 3.0, 4.0, 5.0 and 100 mM potassium-phosphate pH 6.0, including 10 mM β-mercaptoethanol, 5 mM thiourea and 2 mM EDTA. The homogenate was centrifuged at 10,000 x g for 30 min at 4 °C and supernatant was used to assay enzyme activity and protein estimation.

3.3.4. Enzyme extraction and purification

Freshly harvested petals (20 gm) powdered in liquid nitrogen in a chilled pestle and mortar and extracted with two-volumes (w/v) of 100 mM citrate-phosphate buffer (pH 5.0) containing 10 mM β-mercaptoethanol, 5 mM thiourea and 2 mM EDTA with 1% (w/v) insoluble PVPP (Polyclar AT). The homogenate was centrifuged at 10,000 x g at 4 °C (30 min), clear supernatant (40 ml) was collected and assayed for enzyme activity and subjected to purification. All purification steps were carried out at 0-4 °C, unless specified otherwise.

3.3.4.1.. Ammonium sulfate fractional precipitation

The supernatant was subjected to differential solid ammonium sulfate precipitation and the protein fraction precipitating between 10-40% ammonium sulfate saturation was collected by centrifugation (10,000 x g, 10 min), dissolved in 10 mM potassium-phosphate buffer pH 6.0 (4.0 ml) and proceeded to anion molecular weight exclusion chromatography.

3.3.4.2. Size exclusion chromatography through Sephacryl S-200-HR

The ammonium sulfate pellet was loaded on to a Sephacryl S-200-HR (1.4 x 40 cm; void volume 18 ml; flow rate 0.5 ml min^{-1}) and run with pre-equilibration buffer 10 mM potassium-phosphate pH 6.0. Fractions (2.0 ml) collected were screened for native protein at A_{280} as well as β-glucosidase

activity and the catalytic peak of enzyme activity was pooled to run on anion exchange chromatography.

3.3.4.3. Anion exchange chromatography on Q-Sepharose column

Anion exchange chromatography was performed on a Q-Sepharose column (1.5 x 10 cm; flow rate 0.75 ml min^{-1}) pre-equilibrated with 10 mM potassium-phosphate buffer (pH 6.0), sequentially eluted with buffer followed by the buffer containing 0.1 M NaCl and 2.0 ml fractions were collected, monitored for protein (A_{280}) and the enzyme activity. The peak of activity was pooled and proceeded to further step of purification on cation exchange chromatography.

3.3.4.4. Cation exchange chromatography on S-Sepharose column

The enzyme preparation of Q-Sepharose column was directly subjected to cation exchange chromatography on S-Sepharose column (1.5 x 10 cm; flow rate 0.75 ml min^{-1}). The column was pre-equilibrated with 10 mM potassium-phosphate buffer (pH 6.0) and sequentially eluted with buffer followed by the buffer containing 0.2 M NaCl and finally 0.4 M NaCl. Fractions (2.0 ml) collected were monitored for protein (A_{280}) as well as enzyme activity and the fractions with highest specific activity were subjected to SDS-PAGE analysis and used for physico-kinetic characterization.

3.3.5. Enzyme assay

β-Glucosidase activity was assayed by the modified method of Stevens *et al.* (1993) (as detailed in head **3.1.5.**) using *p*-nitrophenyl-β-D-glucopyranside as substrate in assay buffer 100 mM citrate-phosphate pH 5.5 and optimal incubation temperature 40 °C.

3.3.6. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was run in a Mini-PROTEAN-II gel electrophoresis system (BioRad) BioRad) using modified method of Doucet and Trifaro (1988) (as detailed in head **3.1.6.**).

3.3.7. Protein estimation

Protein estimation was done by the method of Lowry *et al.* (1951) using bovine serum albumin as reference standard.

3.3.8. Native molecular weight and subunit composition

Native and subunit molecular weight of the purified enzyme were deciphered from the Sephacryl S-200-HR column chromatography and SDS-PAGE subsequently. The Sephacryl S-200-HR column was calibrated with standard native protein molecular weight markers (M/s Amersham Pharmacia Biotech.). One mg each of bovine serum albumin (67,000), ovalbumin (43,000) and chymotrypsin (25,000) were loaded and eluted with 10 mM phosphate buffer pH 6.0 and a calibration plot was drawn between the $\log_{10}$ molecular weight and average elution volume (K_{av}). The SDS-PAGE for purification samples was co-electrophoresed with standard protein molecular weight markers (M/s Banglore Genie) which contained phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,100), and lysozyme (14,300) (M/s Biogene, USA). The molecular weights of the two polypeptides observed in the final enzyme preparation were computed (74.1 kDa and 67.5 kDa) and compared with native molecular weight as well as analyzed for limited proteomics (as detailed ahead under section **3.8**).

3.3.9. pH optima and pH stability

The pH optimum of the enzyme was acquired from the assay mixtures with pH range 3.0 to 9.0 in assay buffer 100 mM citrate-phosphate containing 5

μL enzyme preparation and 2.5 mM substrate (*p*NPG). The enzyme activity was recorded at spectrophotometrically at 405 nm. To find the pH stability of the enzyme a fraction (1.0 ml) of enzyme preparation was passed though Sephadex G-25 column and desalted against water so that pH remain neutral. The enzyme was mixed with 100 mM citrate phosphate buffer of different pH in equal volume to a final concentration of two fold diluted enzyme preparation in 50 mM buffer of different experimental pH. The pH stability experiment was done with this preparation using 10 μL enzyme aliquot in 200 μL assay mixture containing 2.5 mM substrate at optimum reaction conditions.

3.3.10. Thermostability and thermotropic behaviour

The optimal activity of enzyme for temperature was estimated by assaying activity of standard reaction mixture at different incubation temperatures ranging from 4-80 °C. For thermostability the enzyme preparation was kept at specified experimental temperatures for fixed time duration and activity was assayed using 10 μL enzyme aliquot in 200 μL assay mixture containing 2.5 mM substrate at optimum assay conditions.

3.3.11. Substrate saturation kinetics

The catalytic functionality of the enzyme was monitored by measuring enzyme activity at different substrate (*p*-nitrophenyl-β-D-glucopyranoside) concentrations in the range of 0.05 to 0.70 mM). One ml assay mixture contained 5 μL enzyme at optimal reaction conditions for 5 min and activity was recorded. The K_m and V_{max} functions were discerned from the kinetics plots drawn for enzyme catalysis *versus* substrate concentration using GraphPad™ scientific software in non-linear regression mode.

3.3.12. Substrate specificity

To construe the substrate specificity of enzyme different substrate analogues *p*-nitrophenyl-β-D-glucopyranoside (β-*p*NPGlu), *p*-nitrophenyl-α-D-glucopyranoside (α-*p*NPGlu), *o*-nitrophenyl-β-D-glucopyranoside (β-*o*NPGlu), *p*-nitrophenyl-β-D-galactopyranoside (β-*p*NPGal), *p*-nitrophenyl-α-D-galactopyranoside (α-*p*NPGal), *o*-nitrophenyl-β-D-galactopyranoside (β-*o*NPGal), *p*-nitrophenyl-β-D-mannopyranoside (β-*p*NPMan), *p*-nitrophenyl-β-D-xylopyranoside (β-*p*NPXyl), *p*-nitrophenyl-β-D-fucopyranoside (β-*p*NPFuc), and *p*-nitrophenyl-β-D-lactopyranoside (β-*p*NPLac) were used at 2.5 mM concentration in assay mixture containing 5 µL enzyme at optimal assay conditions. After 10 min incubation time the activity was recorded.

3.3.13. Inhibition kinetics

The inhibition analysis of D-gluconic acid lactone on enzyme was estimated by applying a range of 0.5 to 60 mM inhibitor concentration in assay mixture (1.0 ml) containing 2.5 mM substrate (*p*NPG) and 5 µL enzyme preparation in 100 mM citrate-phosphate buffer pH 5.5 at 40 °C for 10 min. The enzyme activity in presence of inhibitor was recorded and the plots were drawn to elaborate the inhibition kinetics for enzyme.

3.3.14. Effect of glucose

The effect of glucose was estimated by adding varying concentrations (0.2-40 mM) of glucose in standard reaction mixture (1.0 ml) which contained 5 µL enzyme with 2.5 mM substrate (*p*NPG) in 100 mM citrate-phosphate buffer pH 5.5. A pre-incubation of 10 min was given to enzyme-glucose state before adding substrate to assay mixture and the enzyme activity was estimated after 10 min incubation at 40 °C.

3.3.15. Effect of cations, chelating agents and thiol directed reagents

To access the effect of several metal ions, chelating agents and other compounds were tested against β-glucosidase activity. Among metals ions mon-, di-, and tri-valent cations like Li^{+}, K^{+}, Na^{+}, Ag^{+}, Hg^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+},Mg^{2+}, Fe^{2+}, and Fe^{3+}; and EDTA and EGTA as chelating agents were used.

Several other compounds like iodoacetate and iodoacetamide were screened. The assay mixture contained each effector in 1.0, 2.5 and 5.0 mM concentrations with 5 μL enzyme in 100 mM citrate-phosphate buffer pH 5.5 at 40 °C. The reaction mixture was incubated for 10 min and before adding substrate a pre-incubation of 10 min was given to enzyme-effector state and activities were recorded.

3.3.16. Effect of organic solvents

Water miscible organic solvents like methanol, ethanol, acetone and dimethyl sulphoxide (DMSO) were tested for effect on enzyme activity at varying concentrations (5, 10, 20, 30, 40, and 50%). One ml assay mixture contained varying solvents concentration with 5 μL enzyme and 2.5 mM substrate (*p*NPG) in 100 mM citrate-phosphate buffer pH 5.5 at 40 °C. Before adding substrate a pre-incubation of 10 min was given to enzyme-solvent state and then activity was recorded after 10 min.

3.3.17. Transglycosylation

For transglycosylation feasibility of *Silybum* β-glucosidase several alcohols and other aglycone molecules used were methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, decanol, geraniol, linalool, menthol, quercetin, withaferin-A, and ursolic acid. The transglycosylation activity was assessed by the method described ahead in head **3.1.18.** with variation in assay buffer 100 mM citrate-phosphate pH 5.5.

3.4. Proteomic analysis of *Silybum marianum* petal β-glucosidase

The lower (67.5 kDa) polypeptide from SDS-PAGE was excised from the Coomassie stained gels and subjected to proteomic analysis (limited peptide sequence fingerprinting) through MALDI-TOF-TOF as detailed below.

3.4.1. Peptide digestion by trypsin

The in-gel digestion of proteins and purification of peptides from plugs was carried out according to the manufacturer's manual. Briefly, protein spots were excised, washed with desalted water, followed by 50% v/v acetonitrile in 25 mM ammonium bicarbonate (pH 8.0), shrunk by dehydration in ACN and vacuum dried. Gel pieces were reswollen in 10–20 μL digestion buffer containing sequencing grade modified 10 μg/mL trypsin (Promega, Madison, WI, USA). After 15 min, 25 μL of 50 mM ammonium bicarbonate was added to keep the gel pieces wet during tryptic cleavage (37 °C, overnight). To extract the peptides, 50% ACN/0.3%TFA solution was added, and the samples were incubated for 15 min and vortexed. The separated liquid was dried under vacuum and the peptides were again dissolved in 10 μL 0.1% TFA. The peptides were purified with C18 reversed-phase minicolumn filled in a micropipette tip, ZipTip C18 (Millipore, Bedford, MA, USA), before MS. The peptide solution was then mixed with a double volume of matrix, CHCA (ABI, Farmingham, USA.) of 10 mg/mL in 50% ACN, 0.1% TFA and spotted onto a MALDI sample plate.

3.4.2. MALDI-TOF/TOF (Matrix Assisted Laser Desorption Ionization-time of flight)

The MS and MS/MS spectrum were acquired in the positive ion mode on MALDI-TOF/TOF Mass Spectrometer (Applied Biosystems 4700 Proteomics Analyzer, Framingham, MA, USA). The instrument was operated in the delayed extraction mode with delay time of 200 ns. Spectra were obtained by

accumulation of 1000 and 4000 consecutive laser shots respectively in MS and MS/MS mode and laser intensity used were in the range of 5000 to 6000. Close external calibration for MS was performed with 4700 Cal Mix (Applied Biosystems) a standard mixture of six peptides des-Arg1-Bradykinin (904.4681), Angiotensin I (1296.6853), Glu1-Fibrinopeptide B (1570.6774), ACTH [clip 1–17] (2093.0867), ACTH [clip 18–39] (2465.1989) and ACTH [clip 7–38] (3657.9294). Mass calibration for MS/MS spectra was performed by fragment masses of precursor Glu1-Fibrinopeptide B (1570.6774). Peak harvesting was carried out using 4000 Series Explorer™ Software (Applied Biosystems). Only baseline corrections were applied to the raw data.

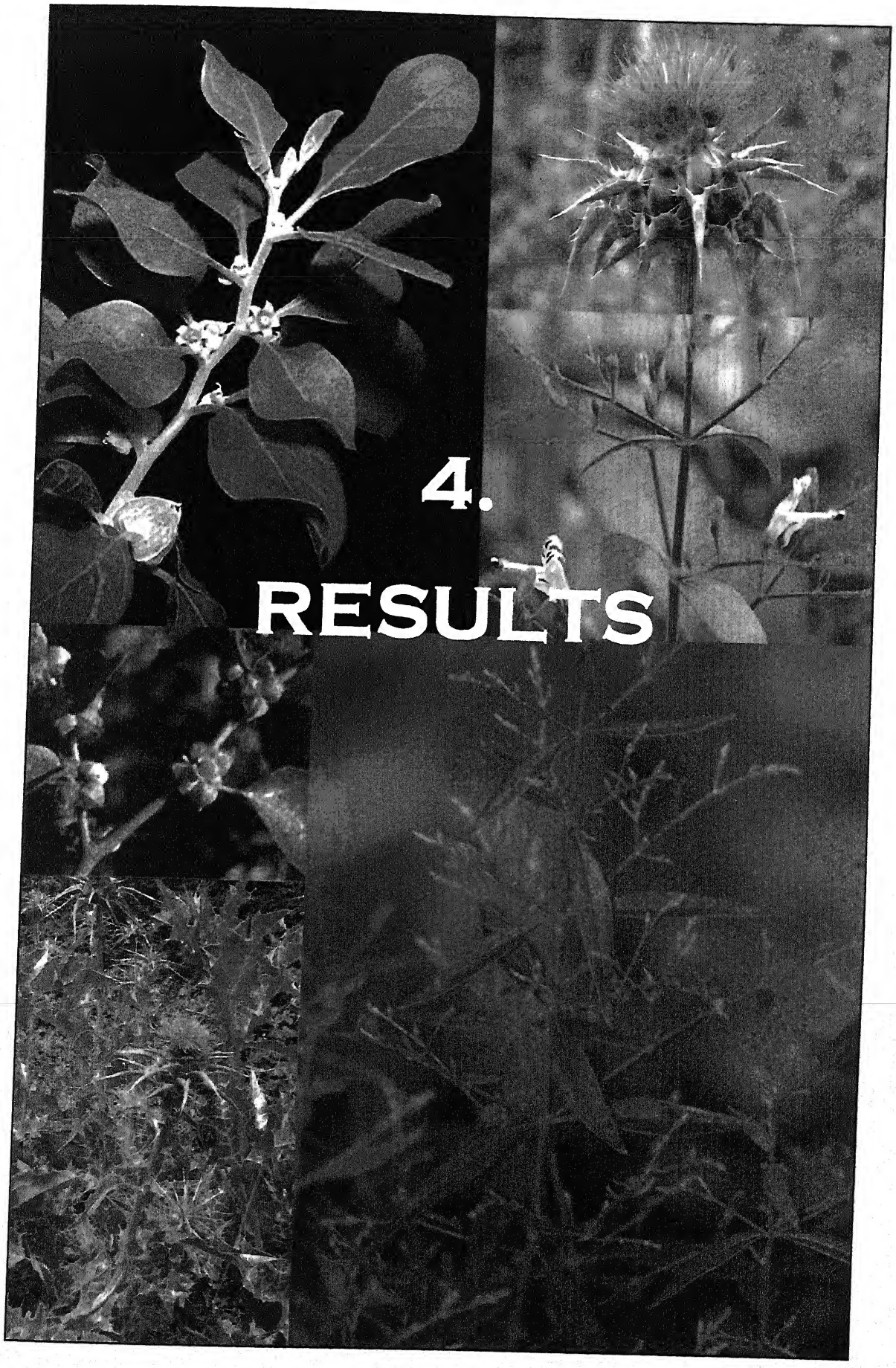
Database searching for protein identifications was performed with mass spectrometry data (MS or MS/MS) using Global Proteome Server v3.5 software (Applied Biosystems) equipped with MASCOT (Matrix Science) search engine. Only monoisotopic masses were used for searching against the Swiss-prot and NCBInr databases with a minimum number of matched masses set at 4. The maximum peptide precursor tolerance was set at 40 ppm and MS/MS fragment tolerance was defined as 0.2 kDa. At the most one missed cleavage for tryptic peptides was allowed, and the modifications accepted were carbamidomethyl cysteines as fixed modification and methionine oxidation as variable modification. MS/MS was performed only in the cases where identification appeared ambiguous with MALDI-TOF-MS data.

3.4.3. Protein sequence BLAST and alignment

The selected fragment sequences were subjected to protein BLAST (The Basic Local Alignment Search Tool) at NCBI (The National Center for Biotechnology Information, USA) [http://www.ncbi.nlm.nih.gov/BLAST/]. The selected significant matching sequences were subjected to multiple sequence alignment (MSA) performed on windows based program ClustalX with other selected β-glucosidases.

3.5. Identification of a fucosyl transferase from *Silybum marianum* petals

The SDS-PAGE gel for purification of *Silybum* petal β-glucosidase showed two protein bands while smaller (67.5 kDa) polypeptide matched with β-glucosidase, the larger one (74.1 kDa) appeared to be unrelated to β-glucosidase by its size estimate compared with native molecular mass. This polypeptide was further characterized for limited fragment sequencing to discern it proteomic identity. The gel plug containing this polypeptide was digested with trypsin and fragment analysis was performed on MALDI-TOF and the resultant *de novo* sequences were BLAST to find out sequence homology (method as described in sections **3.4.1. - 3.4.3.**). Some of the de novo sequences showed strong sequence homology with plant fucosyltransferase, leading to identification of the *Silybum marianum* counterpart.



4. RESULTS

This investigation was focused on purification and characterization of β-glucosidase from three important medicinal plant *Withania somnifera* (Ashwagandha), *Andrographis paniculata* (Kalmegh) and *Silybum marianum* (milk thistle). In all the cases, *a priori* optimization of the recovery of the enzyme from the tissue and optimization of its assay conditions with respect to linearity with time and protein concentration were carried out. The results of investigation on purification and physico-kinetic and functional characteristics of the enzyme from *W. somnifera* leaf, *A. paniculata* leaf and *S. marianum* flowers (petals) are presented sequentially in this chapter of the dissertation.

4.1. *Withania somnifera* leaf β-glucosidase

The purification of β-glucosidase from *Withania somnifera* leaf was achieved through a combination of preparation of activity enriched crude homogenate, differential protein precipitation and column chromatography through different matrices.

4.1.1. Leaf β-glucosidase extraction

Quantitatively appropriate and activity enriched extraction of enzyme is an important facet of achieving isolation of purified enzyme in good yield and purity. The crude homogenate prepared in the buffer of pH 6.0 showed highest β-glucosidase activity while only 33% activity was obtained at pH 2.5. However, total protein content in the extract prepared at pH 6.0 was found to be highest whereas 90% lower protein was measured at pH 2.5. Thus, the tissue extract prepared at pH 2.5 was most enriched in the enzyme specific activity (Table 1). Therefore, for enzyme purification purpose the crude homogenate prepared with the extraction buffer of pH 2.5 was used.

Table 1: β-Glucosidase yield and enrichment in the enzyme extracts of *Withania somnifera* leaf prepared with buffers of different pH.

Extraction buffer pH	Activity (IU / gm f.wt.t.)	Protein (mg / gm f.wt.t.)	Specific Activity (IU / mg)
2.5	0.033	0.660	0.050
3.0	0.052	2.334	0.022
6.0	0.100	6.072	0.016

4.1.2. β-Glucosidase purification

Differential precipitation of the crude enzyme with solid ammonium sulphate in 40-60% saturation resulted in about 10 fold purification with 35% recovery (Fig. 1A). The activity enrichment could be enhanced to 16 fold (with 21% recovery) on its chromatography through a cation exchange (S-Sepharose) column (Table 2). The elution patterns of protein and β-glucosidase activity from the columns (under 1 M KCl in 10 mM phosphate buffer pH 6.0) are presented in Fig. 1B. Further, on chromatiography through hydrophobic interaction chromatography (octyl-Sepharose CL-4B), the activity was eluted under 10 mM potassium-phosphate buffer (pH 6.0) containing 1.0 M ammonium sulfate and this step resulted in 63 fold purification with more than 15% recovery (Fig. 1C). Subsequent chromatography (elution with 0.2 M NaCl in 10 mM phosphate buffer pH 6.0) of the enzyme preparation through an anion exchange column (Q-Sepharose) gave 8% recovery of the enzyme activity with 457 fold purification (Fig. 1D). A repeated cation exchanger chromatography through S-Sepharose (Fig. 1E) revealed 368 fold purification with 0.5% recovery (Table 2). The comparative polypeptide compositions of the enzyme preparations through SDS-PAGE followed by sensitive silver staining revealed that the final enzyme preparation was purified to apparent homogeneity (Fig. 2). The homogeneity

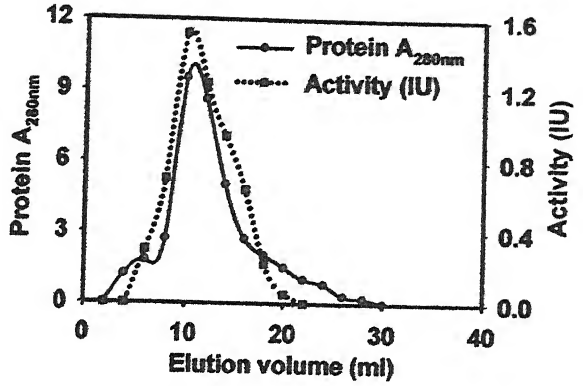
purified enzyme preparation was subjected to physico-kinetic characterization of the enzyme.

Table 2: Purification profile of *Withania somnifera* leaf β-glucosidase.

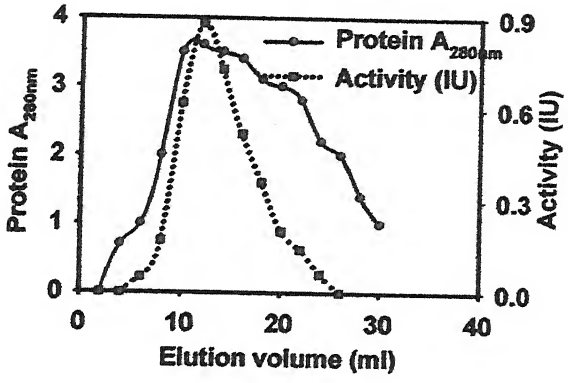
Purification Step	Volume (ml)	Total Activity (IU)	Total Protein (mg)	Specific Activity	Purification Fold	Recovery (%)
Crude homogenate	1510	51	2588.14	0.019	1	100
Sephadex G-25-I	12	18	93.096	0.193	10.1	35.2
S-Sepharose-I	12	11	35.556	0.309	16.2	21.5
octyl-Sepharose CL-4B	10	8	6.650	1.203	63.3	15.6
Sephadex G-25-II	4	6	6.324	0.948	49.8	11.7
Q-Sepharose	10	4	0.460	8.695	457.63	7.8
S-Sepharose-II	2	0.28	0.040*	7.000	368.42**	0.5

*over-estimated protein due to direct measurement rather than TCA precipitation has given relatively underestimation** of fold purification in the fraction compared to the preceding step preparation.

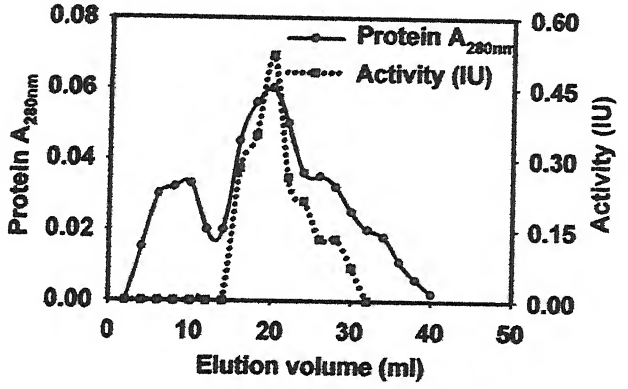
A



B



C



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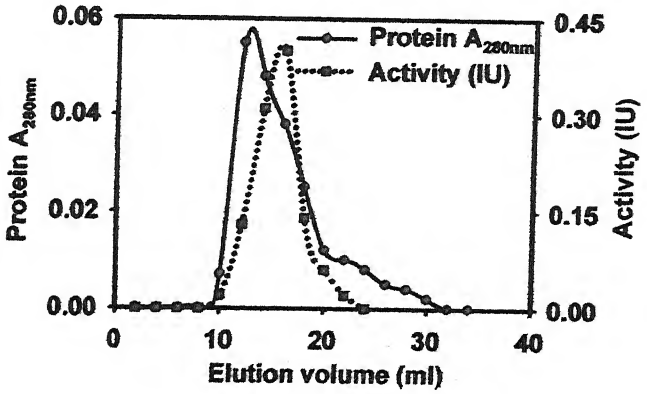


Figure 1: Protein and activity profile of β-glucosidase at different purification steps. (A) S-Sepharose-I, (B) octyl-Sepharose CL-4B, (C) Q-Sepharose, (D) S-Sepharose-II.

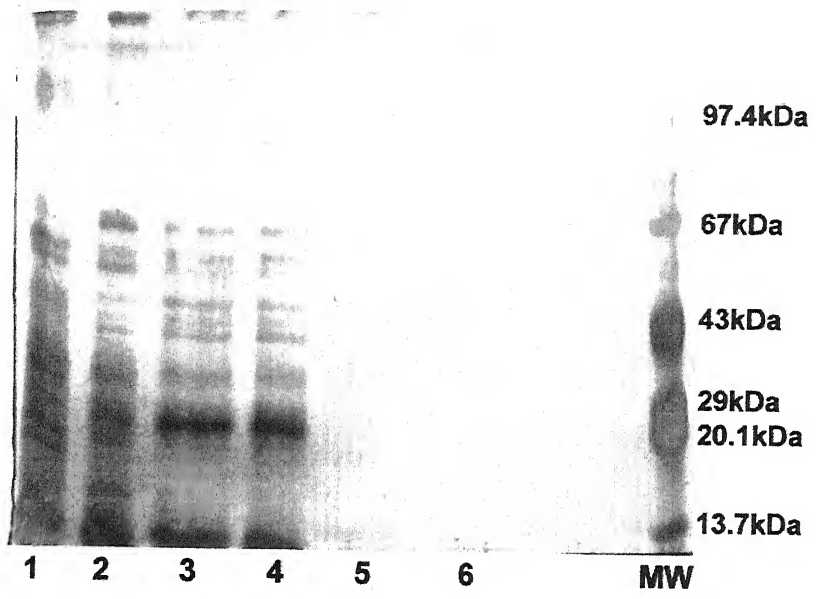


Figure 2: SDS-PAGE (silver stain) for *Withania somnifera* β-glucosidase at different stages of purification. Lanes: 1, crude homogenate; 2, ammonium sulphate; 3, S-Sepharose-I; 4, octyl-Sepharose CL-4B; 5, Q-Sepharose; 6, S-Sepharose-II Fraction-9, MW.

4.1.3. Native molecular weight and subunit composition

Native molecular weight of the purified enzyme was estimated by its chromatography through Sephadex G-75 column. The void volume of the column was determined (28 ml) and the column was calibrated with standards of native protein molecular weight markers. A plot (Fig. 3A) of portioning coefficient (K_{av}) *versus* $\log_{10}$ MW drawn. The elution pattern of the enzyme extract from the calibrated column revealed its native molecular weight to be about 50 kDa. Silver stained SDS-PAGE gel of the enzyme preparation had a single polypeptide band (Fig. 2) of about 50 kDa, as computed from the calibration curve drawn by plotting the molecular weight of standard polypetides *versus* their migration distance (Fig. 3B).

4.1.4. pH stability and pH optimum

The assays carried out at different pH showed that the enzyme had acidic pH optimum (4.8) for the catalytic activity with half maximal activities displayed at pH 4.0 and 7.0 (Fig. 4).

Stability of enzyme activity at different pH was observed by incubation of the enzyme preparation with 100 mM citrate-phosphate buffer of pH 3.0, 4.0, 5.0, 6.0, 7.0 and 100 mM Tris-buffer of pH 8.0, 9.0. The relative pattern of activity after incubation at different pH is presented in Fig. 5. The data revealed that the enzyme had maximal stability at pH 6.0 with better levels of retention of activity in the acidic side.

4.1.5. *p*I (Preparative isoelectric focusing)

On Rotafor Cell preparative isoelectric focusing of *Withania somnifera* leaf β-glucosidase, the zone of prominent enzyme activity was noted in the range of 8.7 to 8.8 giving an estimate of its isoelectric point (*p*I) value of 8.7 (Fig. 6).

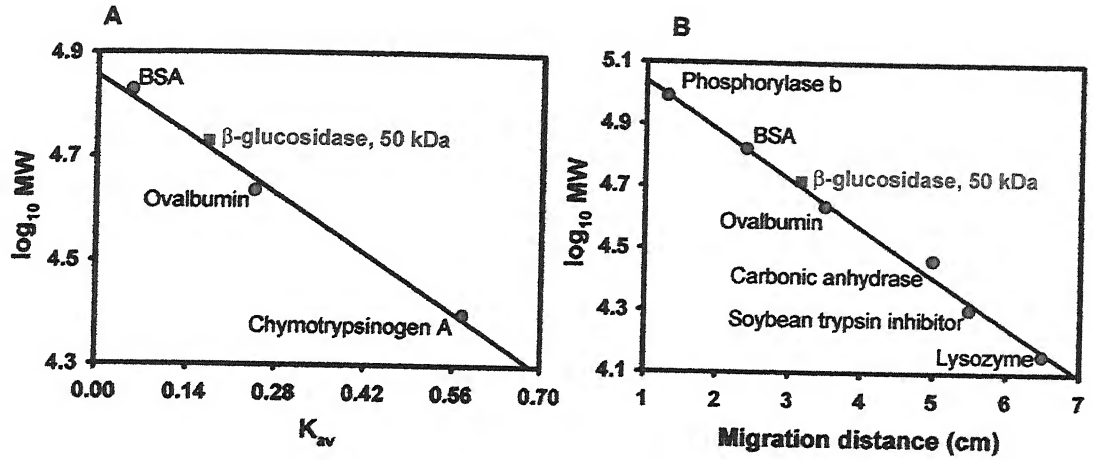


Figure 3: (A) Native MW calibration plot from Sephadex G-75, (B) Subunit MW calibration plot from SDS-PAGE.

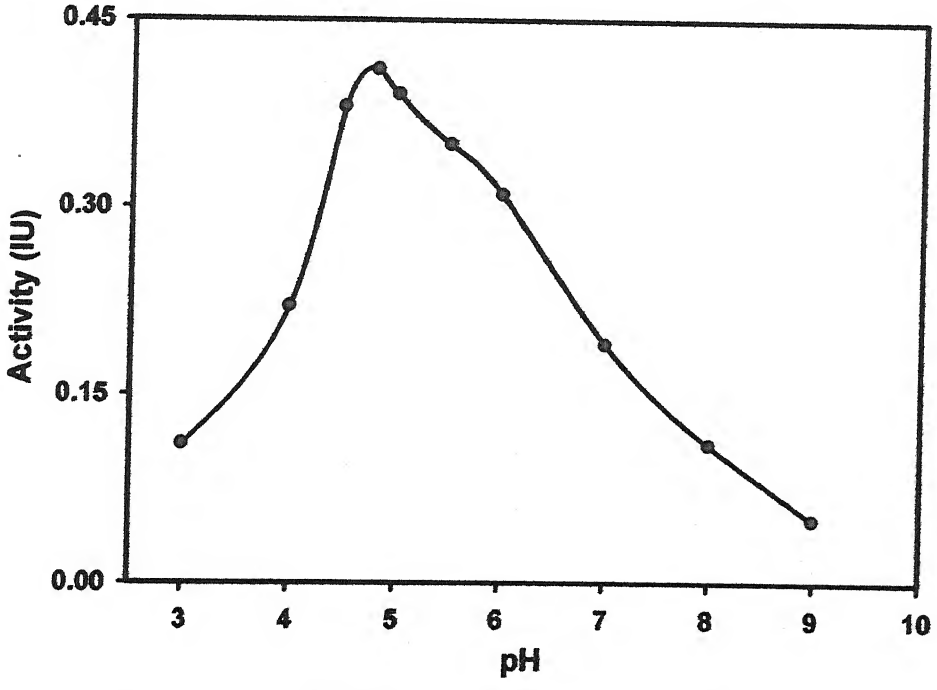


Figure 4: pH optimization of *Withania somnifera* β-glucosidase

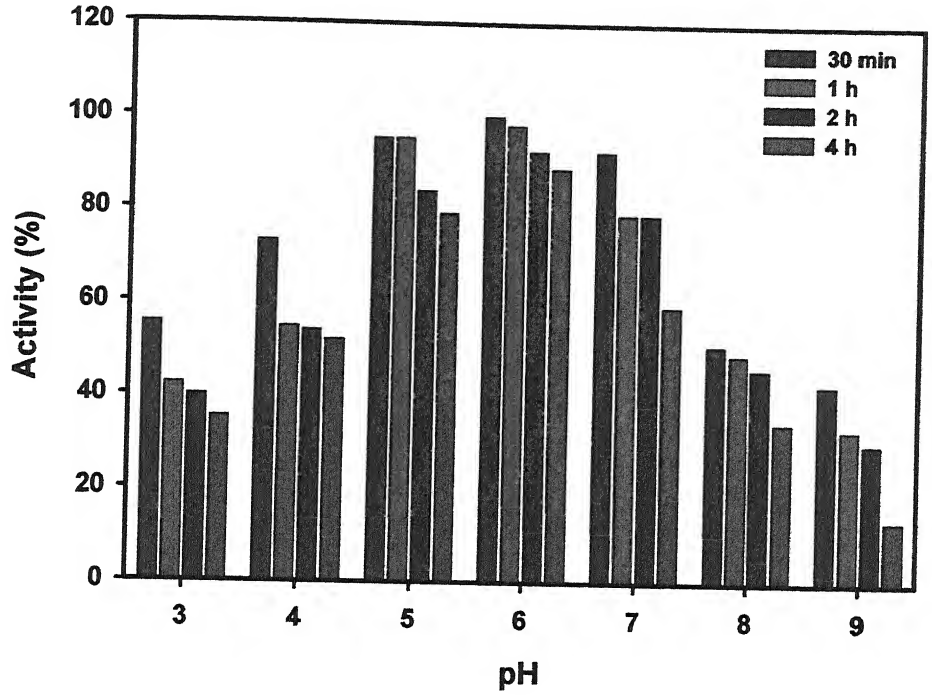


Figure 5: pH stability profile of *Withania somnifera* β-glucosidase.

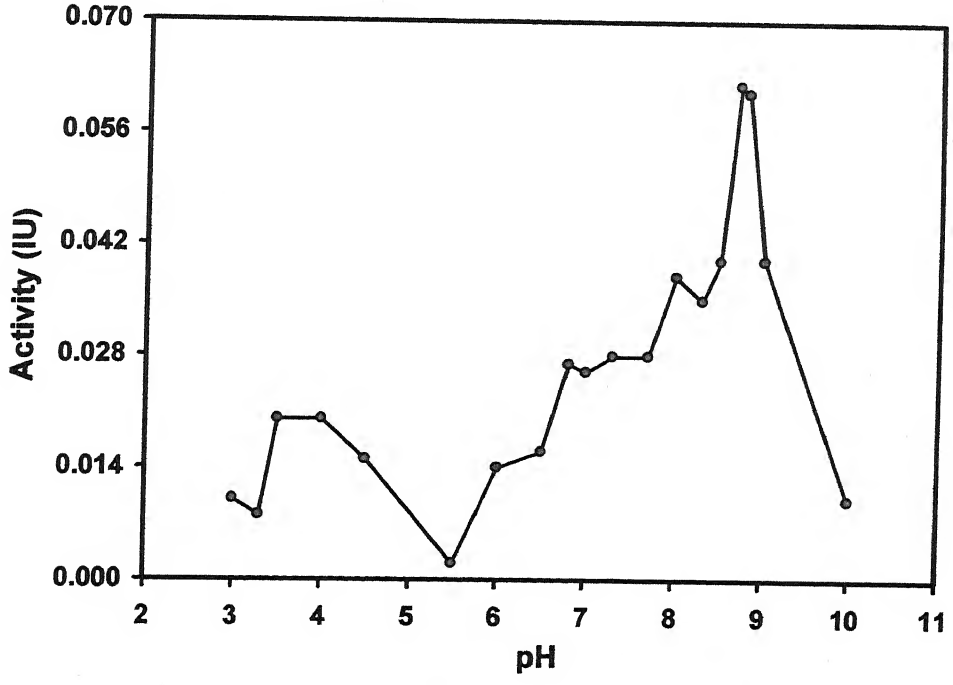


Figure 6: Isoelectric focusing of *Withania somnifera* β-glucosidase.

4.1.6. Thermostability and thermotropic behaviour

The temperature of optimal activity of the enzyme was noted to be 40 °C from the comparative rates of the catalytic reaction assays run at different temperatures (Fig. 7). For the estimation of energy of activation (E_a) an Arrhenius plot was drawn between $\log_{10}$ activity *versus* inverse of absolute (°K) temperature (1/T) (Fig. 7*inset*). The Arrhenius plot of thermotropic behaviour of the enzyme was a single straight line that led to computation of its activation energy value of 12.4 kCal.Mol^{-1}.

The enzyme stability was analyzed by its incubation at different temperatures followed by estimation of the residual activity in the preparation. The temperature of incubation (storage) *versus* the catalytic activity residual after the incubation (Fig. 8) revealed the enzyme to be stable at only low temperatures (4-10 °C).

4.1.7. Substrate saturation and catalysis kinetics

The plots of kinetics of catalysis were drawn to determine K_m and V_{max}. The substrate saturation curve of the enzyme for β-*p*NPG was normal hyperbolic (Fig. 9) implying that the enzyme followed normal Michaelis-Menten kinetics of reaction catalysis. The reciprocal plots (Fig. 9*inset*) gave an estimate of K_m and V_{max} values of 0.19 mM and 22.85 IU, respectively for β-*p*NPG.

4.1.8. Substrate specificity

The enzyme substrate specificity was tested was tested against various artificial substrates with different types of linkages (β or α), different glyco-moieties and alternate position of the substituent group (-NO_2) on the aglycone moiety. These included β-linked sugars conjugates like *p*-nitrophenyl-β-D-glucopyranoside (β-*p*NPGlu), *p*-nitrophenyl-β-D-galactopyranoside (β-*p*NPGal), *p*-nitrophenyl-β-D-mannopyranoside (β-*p*NPMan), *p*-nitrophenyl-β-D-xylopyranoside (β-*p*NPXyl), *p*-nitrophenyl-β-D-fucopyranoside (β-*p*NPFuc), *p*-

nitrophenyl-β-D-lactopyranoside (β-*p*NPLac); α-linked sugars conjugates like *p*-nitrophenyl-α-D-glucopyranoside (α-*p*NPGlu), *p*-nitrophenyl-α-D-galactopyranoside (α-*p*NPGal); aglycone substituent group position variants like *o*-nitrophenyl-β-D-glucopyranoside (β-*o*NPGlu) and *o*-nitrophenyl-β-D-galactopyranoside (β-*o*NPGal). Interestingly, the *W. somnifera* leaf β-glucosidase displayed significant deglycosylating actvity only with β-*p*NPGlu, even activity with β-*o*NPGlu was rudimentary (mere 4% as compared to that with β-*p*NPGlu) (Table 3).

Table 3: Substrate specificity of *Withania somnifera* leaf β-glucosidase.

S. No.	Substrate	Activity (%)
1	*p*-nitrophenyl-β-D-glucopyranoside (β-*p*NPGlu)	100
2	*p*-nitrophenyl-α-D-glucopyranoside (α-*p*NPGlu)	0
3	*p*-nitrophenyl-β-D-galactopyranoside (β-*p*NPGal)	0
4	*p*-nitrophenyl-α-D-galactopyranoside (α-*p*NPGal)	0
5	*p*-nitrophenyl-β-D-galactopyranoside (β-*o*NPGal)	0
6	*o*-nitrophenyl-β-D-glucopyranoside (β-*o*NPGlu)	4.4
7	*p*-nitrophenyl-β-D-mannopyranoside (β-*p*NPMan)	0
8	*p*-nitrophenyl-β-D-xylopyranoside (β-*p*NPXyl)	0
9	*p*-nitrophenyl-β-D-fucopyranoside (β-*p*NPFuc)	0
10	*p*-nitrophenyl-β-D-lactopyranoside (β-*p*NPLac)	0

4.1.9. Gluconolactone inhibition kinetics

D-glucono-1,5-lactone acted as strong inhibitor for enzyme activity with >50% inhibition of the activity at 100 μM concentration. Almost complete (>90%) inhibition was noted at 1.0 mM (Fig. 10).

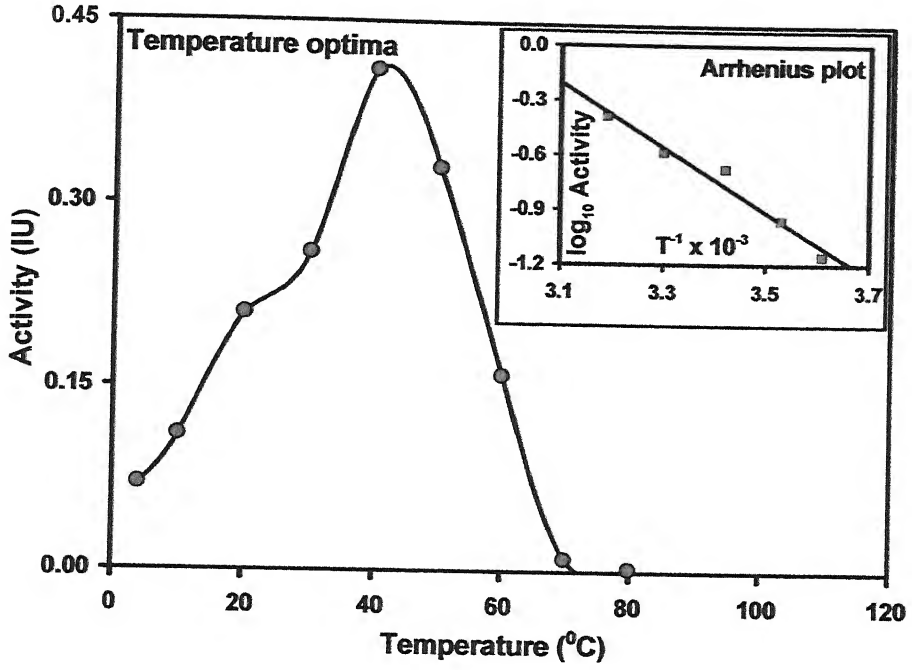


Figure 7: Temperature optima and thermotropic behaviour of *Withania somnifera* β-glucosidase.

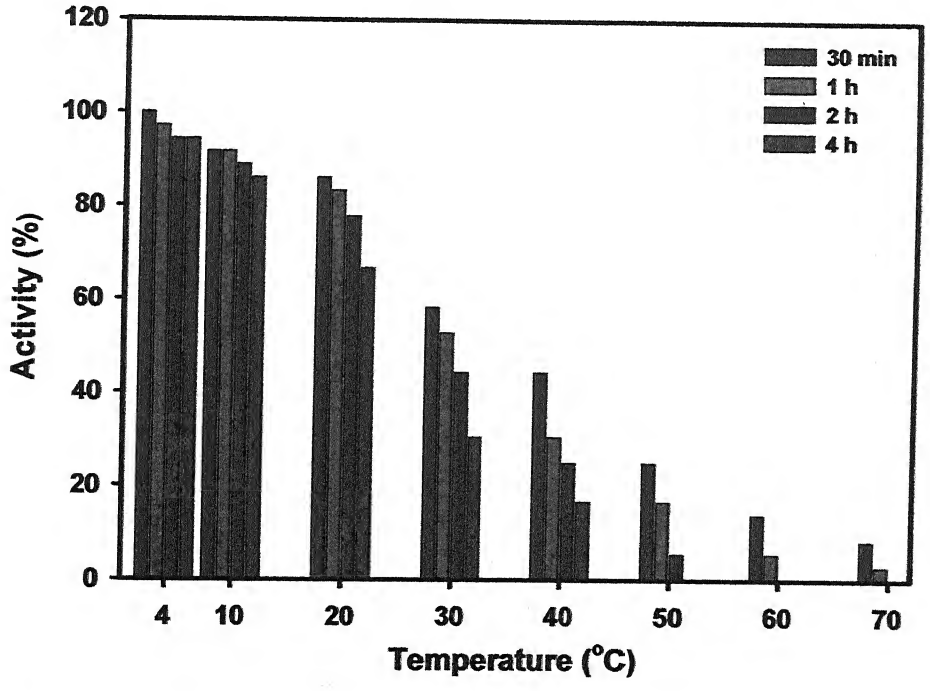


Figure 8: Temperature stability profile of *Withania somnifera* β-glucosidase.

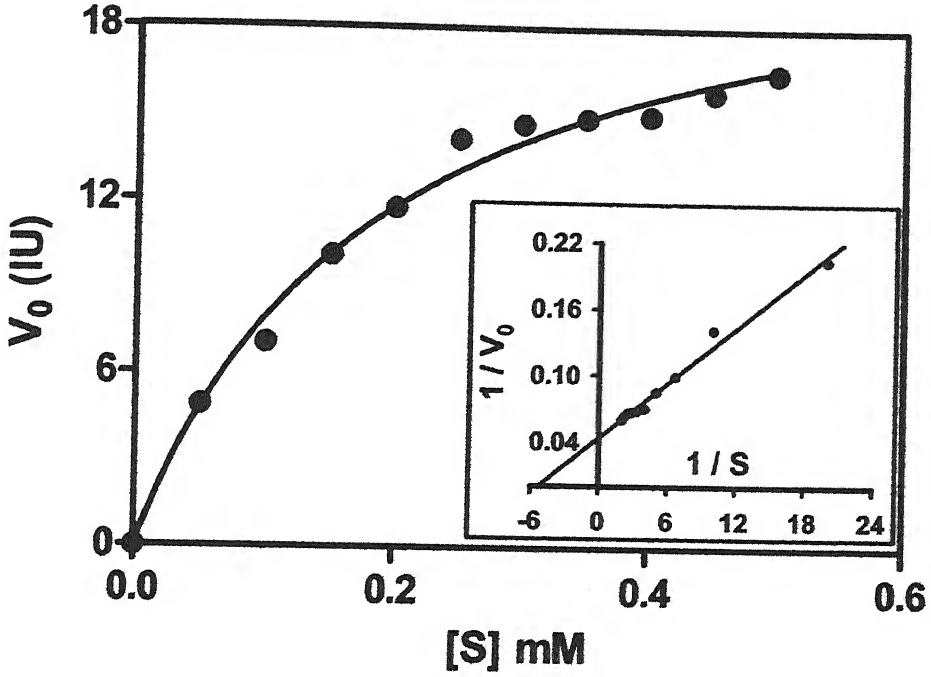


Figure 9: Substrate (*p*NPG) saturation kinetics of *Withania somnifera* β-glucosidase.

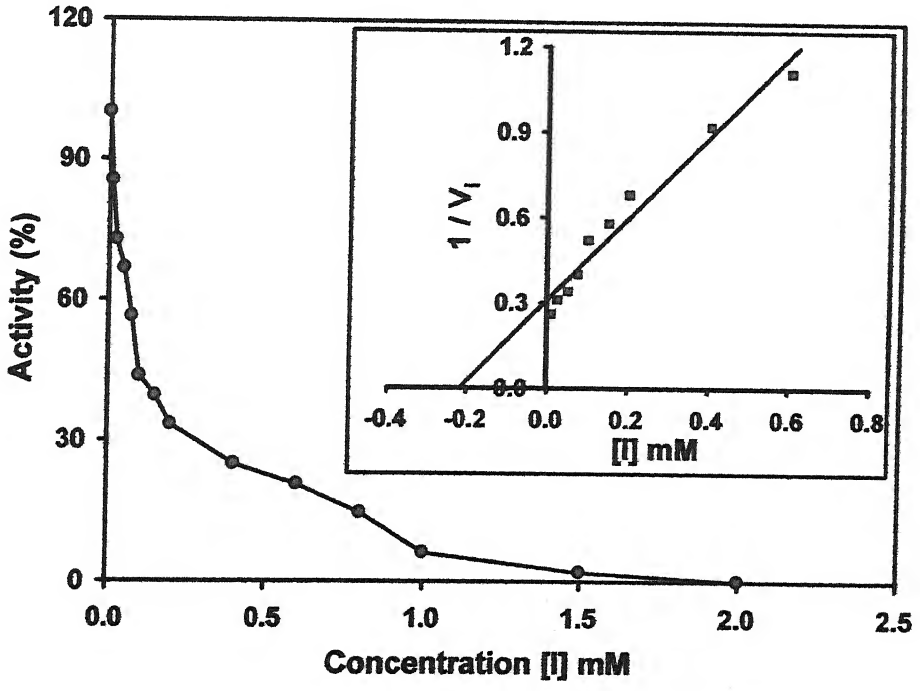


Figure 10: D-gluconolactone inhibition kinetics of *Withania somnifera* β-glucosidase.

Further analysis of the inhibition kinetics revealed that the K_i value of enzyme for D-glucono-1,5-lactone (Fig. 10*inset*), determined from the plot (regression line) of inverse of activity at different inhibitor concentrations ($1/V_i$) *versus* inhibitor concentration (I), was very low (210 μM).

4.1.10. Effect of glucose

Glucose, the end product of the enzyme catalyzed reaction, was found to have no effect on enzyme activity at low concentrations (0.1 to 2.0 mM) but it slightly reduced the activity at higher concentrations (>5 mM). At 40 mM, the final experimental glucose concentration used, the inhibition observed was 24% (Fig. 11).

4.1.11. Effect of cations and chelating agents

Among the metal ions tested, several divalent (Mn^{2+}, Ca^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and Co^{2+}) and monovalent (Li^+, K^+ and Na^+) cations did not have any effect on the catalytic activity at low concentrations (1 mM) but their higher concentrations (5 mM) were slightly inhibitory. But Mg^{2+} and Fe^{2+} showed strong inhibition with the later inhibiting the activity by 75% and 100% at 1.0 mM and >2.5 mM concentrations, respectively. Contrarily, Fe^{3+} activated the catalysis by 1.1 and 1.4 fold at 2.5 mM and 5 mM concentrations, respectively. Hg^{2+} strongly inhibited the enzyme with complete inhibition at 1.0 mM concentration. Similarly, Ag^+ also inhibited upto 42% activity at 1.0 mM and >94% inhibition was observed at 5 mM concentration (Table 4).

Chelating agents like EDTA and EGTA did not influence β-glucosidase activity in *Withania somnifera* by any significant extent as there is no cationic involvement in the catalytic activity of enzyme (Table 4).

Table 4: Effect of metal ions, chelating and other reagents on *Withania somnifera* leaf β-glucosidase.

Effector	Activity (% of control)		
	1 mM	2.5 mM	5 mM
Li^+	90.9	69.8	60.1
K^+	96.8	90.0	89.3
Na^+	100	95.9	93.5
NH_4^+	100	91.9	71.5
Ag^+	57.9	33.0	6.4
Hg^{2+}	0	0	0
Mn^{2+}	93.5	90.0	76.6
Mg^{2+}	76.6	61.3	56.6
Ca^{2+}	100	73.5	60.1
Cu^{2+}	91.9	76.6	38.0
Cd^{2+}	96.8	78.8	66.5
Zn^{2+}	100	65.2	56.6
Co^{2+}	93.5	83.4	53.3
Fe^{2+}	25.0	0	0
Fe^{3+}	98.7	113.4	140.2
EDTA	93.5	88.0	87.5
EGTA	83.4	63.4	53.3
Iodoacetate	86.7	63.4	53.3
Iodoacetamide	120.2	128.8	136.7
PMSF	147.0	127.0	109.5
PCMB	69.8	39.8	19.8
Benzidine	89.5	73.5	56.6

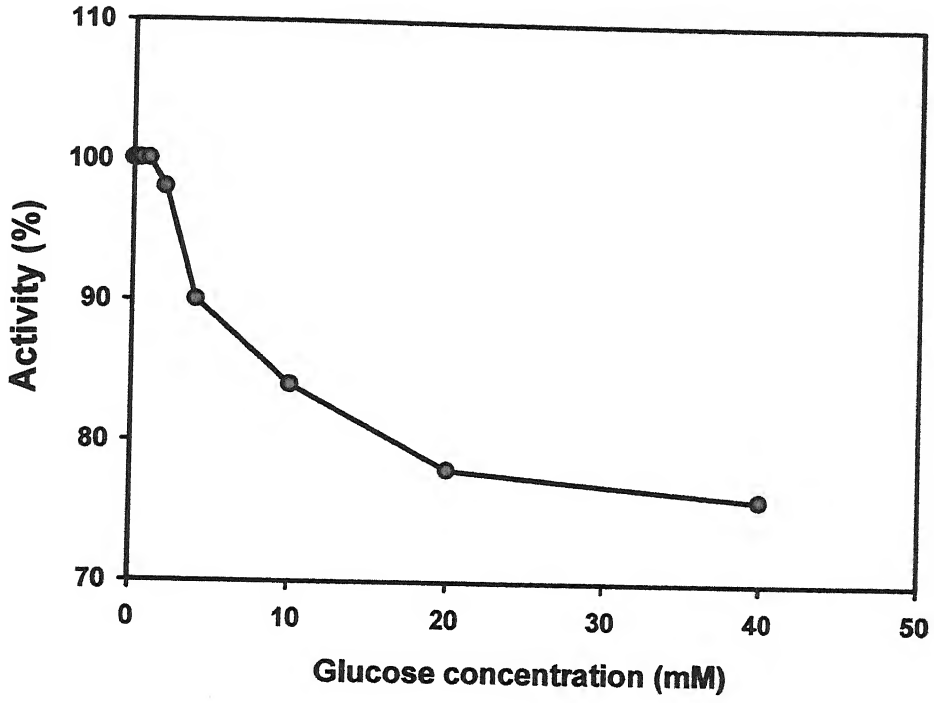


Figure 11: Effect of glucose on *Withania somnifera* β-glucosidase.

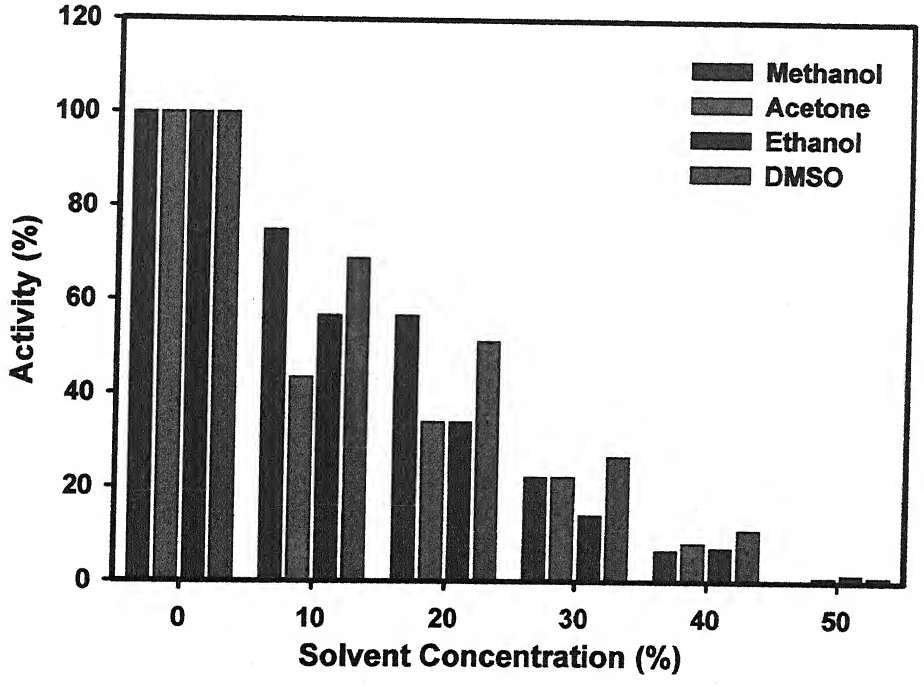


Figure 12: Effect of organic solvents and other alcohols on *Withania somnifera* β-glucosidase.

4.1.12. Effect of thiol directed reagents

Thiol directed reagents like iodoacetamide, iodoacetate, benzidine and *p*-chloromercuribenzoate did not show any significant inhibition of the *Withania somnifera* β-glucosidase activity suggesting lack of –SH groups in the catalysis (Table 4).

4.1.13. Effect of organic solvents

The effect of miscible organic solvents at varying concentrations of (10 to 50%, v/v) (ethanol, methanol, acetone and dimethyl sulphoxide) on β-glucosidase activity were examined. Dimethyl sulphoxide (DMSO) was found to reduce the *Withania somnifera* β-glucosidase hydrolytic activity by about 30% activity at 10% and almost completely at 50% concentrations (Fig. 12). Almost identical pattern was observed with other solvents except that inhibition values with acetone were higher.

4.1.14. Transglycosylation

The transglycosylating catalytic activity of the *Withania somnifera* β-glucosidase was assessed using different glucosyl donors and acceptors in the assay mixture (Fig. 13) and thin layer chromatographic (T.L.C.) analyzing the reaction mixture at the end of reaction mixture to visualize any glycoside (polar) formation. The catalytic reaction products were monitored and compared with appropriate controls. Glycoside formation was observed (R_f 0.27 i.e. slightly above *p*-nitrophenyl-β-D-glucopyranoside) only with geraniol as the aglycone acceptor and *p*NPG as the glycosyl donor (Fig. 14). Whereas, the transglycosylation reaction using cellobiose as the glucosyl donor did not result into any glycosylating reaction with any of the substrates.

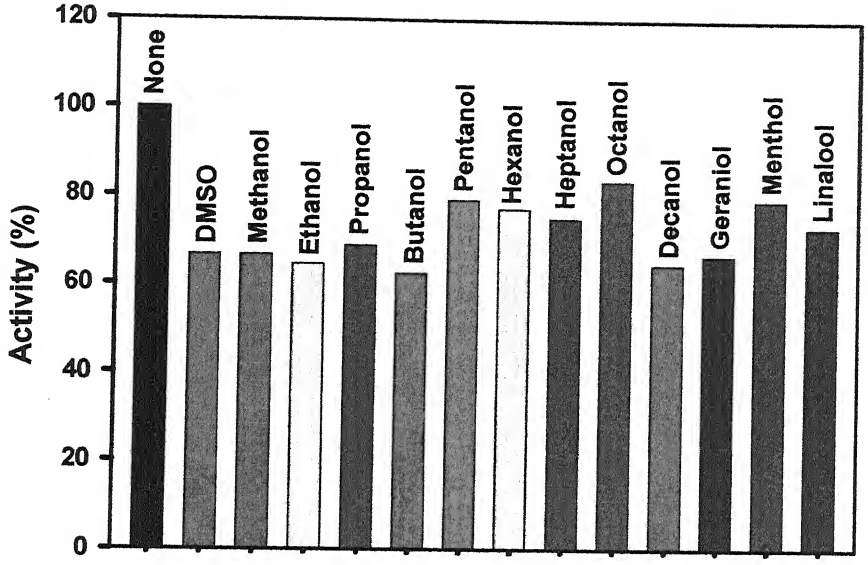


Figure 13: Effect of glucosyl acceptors in the assay mixture on *Withania somnifera* β-glucosidase.

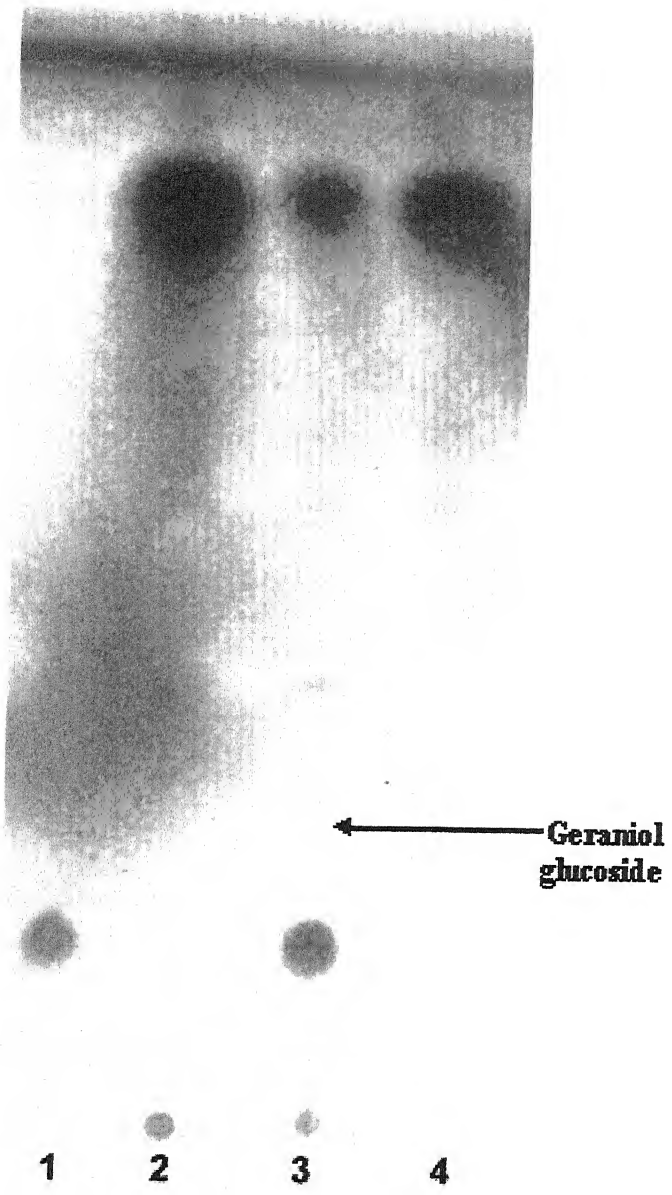


Figure 14: TLC analysis of transglucosylation reaction catalyzed by *Withania somnifera* β-glucosidase. Lanes: 1, *p*-nitrophenyl β-D-glucopyranoside standard; 2, geraniol standard; 3, reaction mixture with pNPG; 4, reaction mixture with cellobiose.

4.2. *Andrographis paniculata* leaf β-glucosidase

The purification of β-glucosidase from *Andrographis paniculata* leaf was achieved through a combination of protein precipitation and column chromatography through different matrices, using extraction conditions *a priori* optimized for suitable yield and optimal specific activity. The purified enzyme preparation was used for its characterization with respect to physical, kinetic and functional attributes. This study has identified, isolated and characterized a novel gluconolactone inhibition insensitive β-glucosidase from *Andrographis paniculata* leaf.

4.2.1. Enzyme isolation optimization

The enzyme extraction was optimized at the level of extraction buffer pH and thus a high specific activity content preparation with suitable yield was obtained for subjecting to the purification process. The enzyme extracted in a buffer of pH 2.0 had lowest activity *per se* but the highest specific activity. Contrarily, enzyme isolated at pH 6.0 had highest enzyme activity and relatively lower specific activity. However, use of pH 3.0 extraction buffer led to moderately enriched specific activity with suitable yield of the enzyme to subject to further purification (Table 5). Therefore, extraction buffer (100 mM citrate-phosphate) pH 3.0 was used for the enzyme isolation.

Table 5: β-Glucosidase isolation optimization with reference to extraction pH.

Extraction buffer pH	Activity (IU / gm f.wt.t.)	Protein (mg / gm f.wt.t.)	Specific Activity (IU / mg)
2.0	0.130	1.206	0.107
2.5	0.152	2.464	0.061
3.0	0.327	3.841	0.085
4.5	0.436	5.800	0.075
6.0	0.480	5.876	0.081

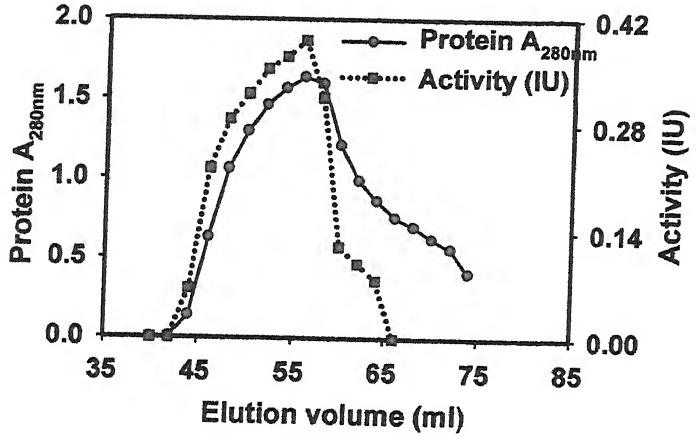
4.2.2. *Andrographis paniculata* leaf β-glucosidase purification

Differential ammonium sulfate precipitation of the crude homogenate followed by gel filtration chromatography through Sephadex G-75 of the protein precipitating under 0-40% saturation resulted in about 2 fold purification with 23% recovery (Fig. 15A; Table 6). Subsequent anion exchange chromatography of the preparation through Q-sepharose gave ~4 fold enhancement in specific activity with ~12% recovery (Fig. 15B). Most markedly, purification was achieved (104 fold enrichment of the specific activity with 1.5% recovery) at hydrophobic interaction chromatography through octyl-Sepharose CL-4B (Fig. 15C; Table 6). The fraction with maximal specific activity when checked for purity by silver stained SDS-PAGE was found to be composed of a single polypeptide (Fig. 16A) indicating the purification to be of homogeneity level. The native PAGE of the enzyme followed by *in situ* staining of the enzyme activity revealed the presence of a single β-glucosidase isozyme in the tissue (Fig. 16B). The purified enzyme preparation was used to analyze the physico-kinetic characteristics of the enzyme.

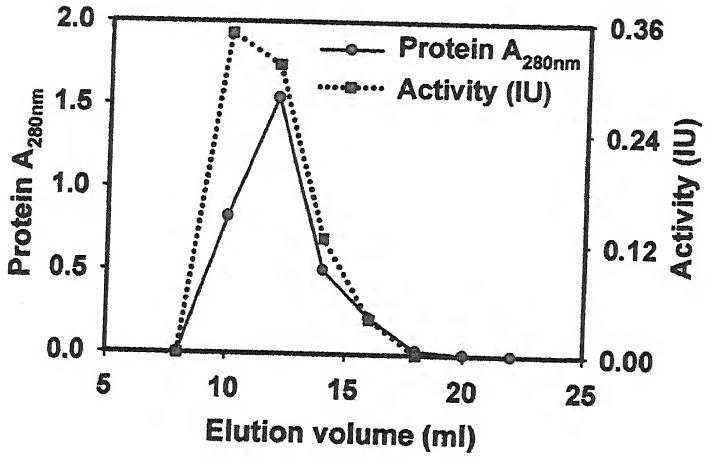
Table 6: Purification profile of *Andrographis paniculata* leaf β-glucosidase.

Purification Step	Volume (ml)	Total Activity (IU)	Total Protein (mg)	Specific Activity	Purification Fold	Recovery (%)
Crude Supernatant	110	33.0	171.16	0.192	1	100
Sephadex G-75	20	7.63	20.70	0.368	1.9	23.1
Q-Sepharose	6	3.92	5.352	0.732	3.8	11.8
octly-Sepharose CL-4B	2	0.52	0.026	20.0	104.1	1.5

A



B



C

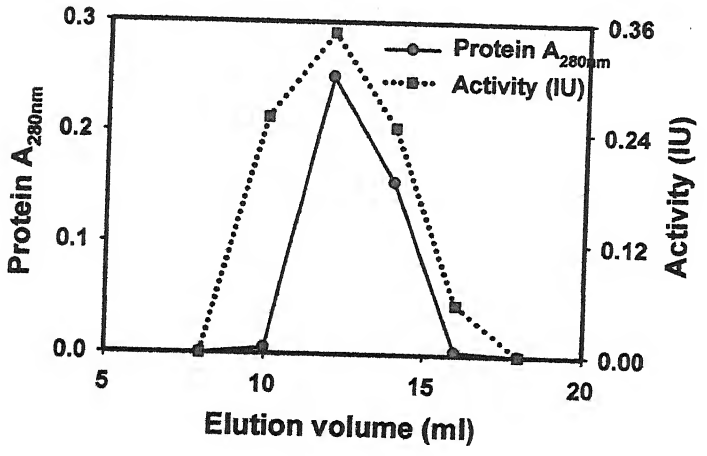


Figure 15: Protein and activity profile of β-glucosidase at different chromatographic purification steps. (A) Sephadex G-75, (B) Q-Sepharose, (C) octyl-Sepharose CL-4B.

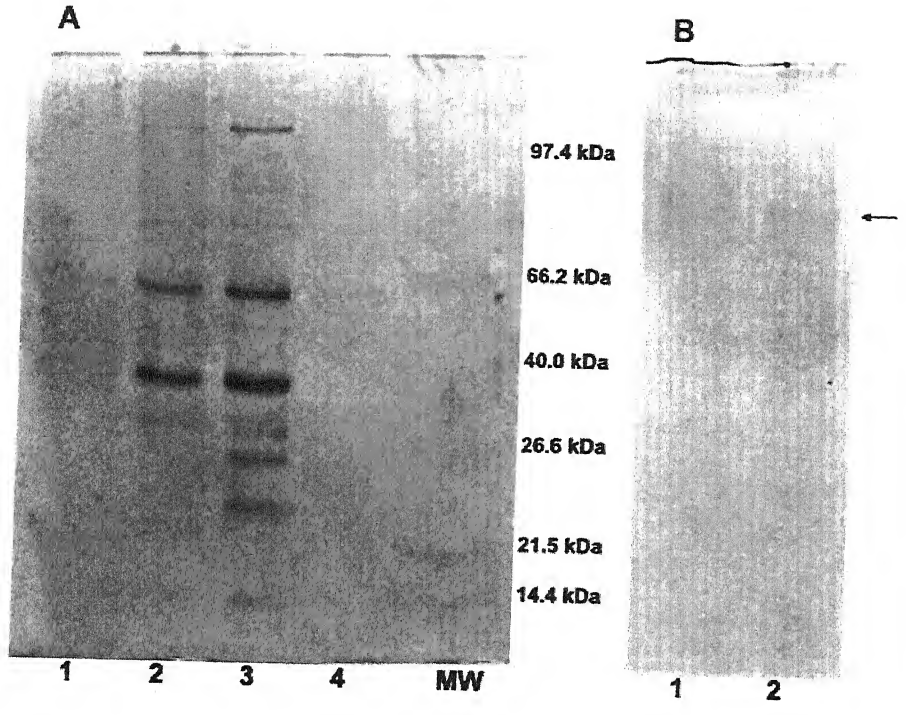


Figure 16: (A) SDS-PAGE (silver stained) for *Andrographis paniculata* leaf β-glucosidase at different stages of purification. Lanes: 1, crude homogenate; 2, Sephadex G-75; 3, Q-Sepharose; 4, Octyl-Sepharose; MW, standard molecular weight marker. (B) Native-PAGE of *Andrographis paniculata* purified β-glucosidase. Lanes: 1, crude homogenate; 2, purified octyl-Sepharose CL-4B fraction.

4.2.3. Native molecular weight and subunit composition

The native molecular weight of *Andrographis panicuata* leaf β-glucosidase was about 60 kDa, as determined by a calibrated size exclusion chromatography through a Sepadex G-75 column using a plot of molecular weight *versus* portioning coefficient (K_{av}) (Fig. 17A). The subunit molecular weight of enzyme was estimated from the migration of standard protein molecular weight markers on SDS-PAGE. A plot was drawn between $\log_{10}$ molecular weight *versus* migration distance (Fig. 17B) and the calibration plot gave an estimate of apparent molecular weight of about 60 kDa for *Andrographis* β-glucosidase subunit. Thus, the enzyme was monomeric in nature.

4.2.4. pH optima and pH stability

The enzyme activity assayed at different pH generated a bell-shaped curve of typical enzyme's pH optima plot (Fig. 18). The pH optima of enzyme was 5.5 (citrate-phosphate buffer).

The analysis of enzyme stability by monitoring the catalytic activity after incubation of the enzyme preparation at different pH (100 mM citrate-phosphate pH 3.0, 4.0, 5.0, 6.07.0 and 100 mM Tris-HCl pH 8.0, 9.0, 10.0) at time intervals of 30 min, 1 h, 2 h, and 4 h of incubation (storage). The data revealed that the enzyme was more stable in the acidic pH range with maximal stability at pH 6.0 (Fig. 19).

4.2.5. Preparative isoelectric focusing

The isoelectric point (*p*I) of *Andrographis paniculata* β-glucosidase was edetrmined from the analyses based on the Rotafor Cell preparative isoelectric focusing. The *p*I of the *Andrographis paniculata* β-glucosidase was 4.0 pH (Fig. 20).

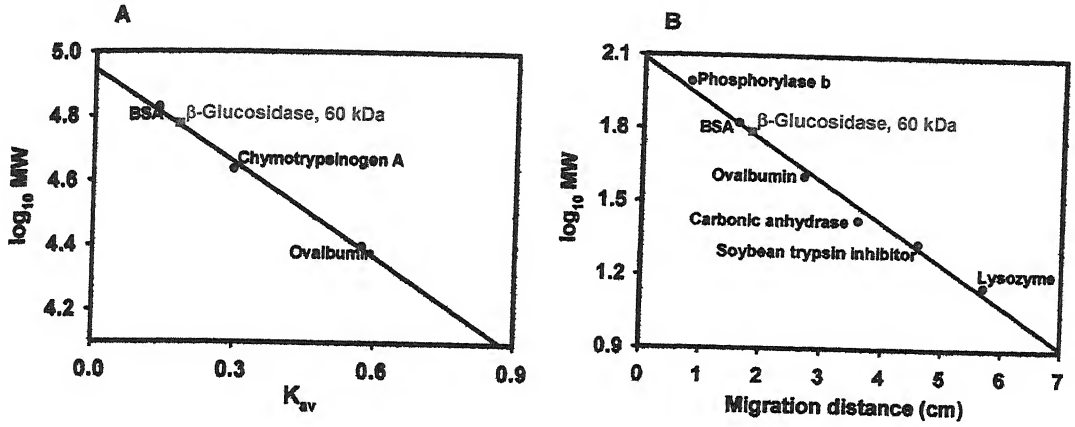


Figure 17: (A) Native MW calibration plot from Sephadex G-75. (B) Subunit MW calibration plot from SDS-PAGE.

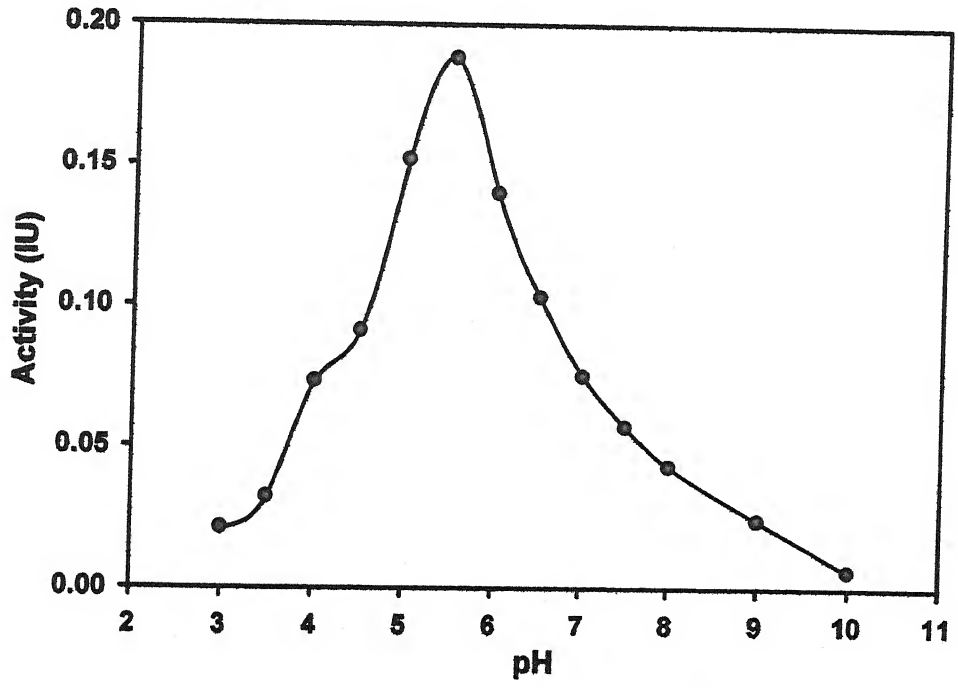


Figure 18: pH optimization of *Andrographis* β-glucosidase

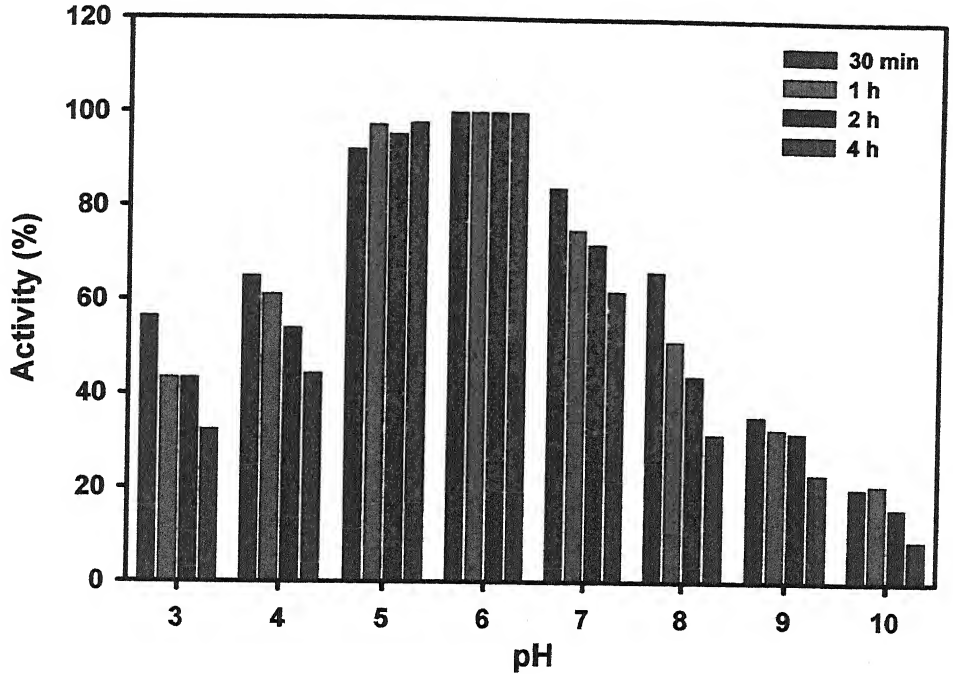


Figure 19: pH stability profile of *Andrographis* β-glucosidase.

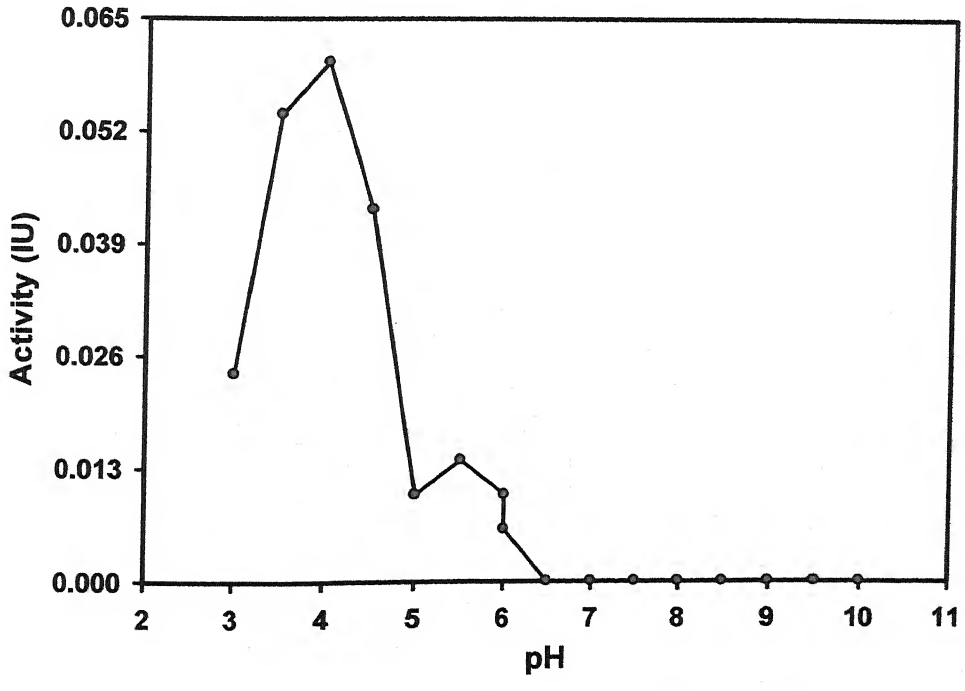


Figure 20: Isoelectric focusing of *Andrographis* β-glucosidase.

4.2.6. Thermostability and thermotropic behaviour

The temperature of optimum activity of enzyme was 55 °C (Fig. 21). Activation energy (E_a) of the enzyme as computed from the Arrhenius plot $\log_{10}$ activity *versus* inverse of absolute temperature (1/T). The plot was a single straight line that gave an estimate of activation energy of 6.8 kCal Mol^{-1} (Fig. 21*inset*).

Thermostability of the enzyme determined by activity assay of the preparation after incubating it at different experimental temperatures for 30 min, 1 h, 2 h, and 4 . The activity profile generated revealed that the enzyme had maximal stability between temperature range 4 to 10 °C (Fig. 22).

4.2.7. Substrate saturation kinetics

The substrate saturation curve for *Andrographis* β-glucosidase was normal hyperbolic plot (Fig. 23), typical for Michaelis-Menten kinetics of reaction catalysis. The Line Weaver-Burk plot (Fig. 23*inset*) gave an estimate of K_m of 0.25 mM and V_{max} of 9.22 IU for β-*p*NPG.

4.2.8. Substrate specificity

The enzyme was screened for its substrate specificity towards artificial substrates with different types of linkages (β or α) and glyco-conjugates including alternate position of the substituent group (-NO_2) on the aglycone moiety. These included several β-linked sugars conjugates and aglycone substituent group position variants (Table 7). Though, the enzyme showed maximal hydrolytic activity towards β-*p*NPGlu but it displayed some activity with β-*p*NP-Mannose (16%) and β-*p*NP-Fucose (10%). It showed negligible activity with β-*p*NP-Xylose and β-*p*NP-Galactose (4-6%) and only rudimentary (1.4%) with α-*p*NP-Glucose among the α-linkage glycosides. Also, little (4%) activity was observed with aglycone nitro-group position variant-position-substituent i.e. *ortho*-NP-Glucose (4%) (Table 7).

Table 7: Substrate specificity of *Andrographis paniculata* β-glucosidase.

S. No.	Substrate	Activity (%)
1	*p*-nitrophenyl-β-D-glucopyranoside (β-*p*NPG)	100
2	*p*-nitrophenyl-α-D-glucopyranoside (α-PNPG)	1.4
3	*o*-nitrophenyl-β-D-glucopyranoside (β-*o*NPG)	4.2
4	*p*-nitrophenyl-β-D-galactopyranoside (β-*p*NPGal)	4.2
5	*p*-nitrophenyl-α-D-galactopyranoside (α-*p*NPGal)	0
6	*o*-nitrophenyl-β-D-galactopyranoside (β-*o*NPGal)	0
7	*p*-nitrophenyl-β-D-mannopyranoside (β-*p*NPMan)	16.5
8	*p*-nitrophenyl-β-D-xylopyranoside (β-*p*NPXyl)	6.1
9	*p*-nitrophenyl-β-D-fucopyranoside (β-*p*NPFuc)	9.9

4.2.9. Gluconolactone Inhibition

The studies on inhibition by D(+)-glucono-1,5 δ-lactone, a common inhibitor for β-glucosidases revealed that the *Andrographis panicuata* β-glucosidase was *almost* insensitive to its inhibitory action. It could inhibit about 50% at very high millimolar (10 mM) concentration. Complete loss of activity was noted at 50 mM D-gluconolactone concentration (Fig. 24).

4.2.10. Effect of glucose

Glucose had moderate inhibitory effect on *Andrographis* enzyme with a negligible (8-10%) inhibition at concentrations upto 0.5 mM. The inhibition remained very low (16-20%) even when glucose concentration in the assay mixture was enhanced to upto 20 mM. Even at 40 mM glucose, the inhibition was merely 23% (Fig. 25).

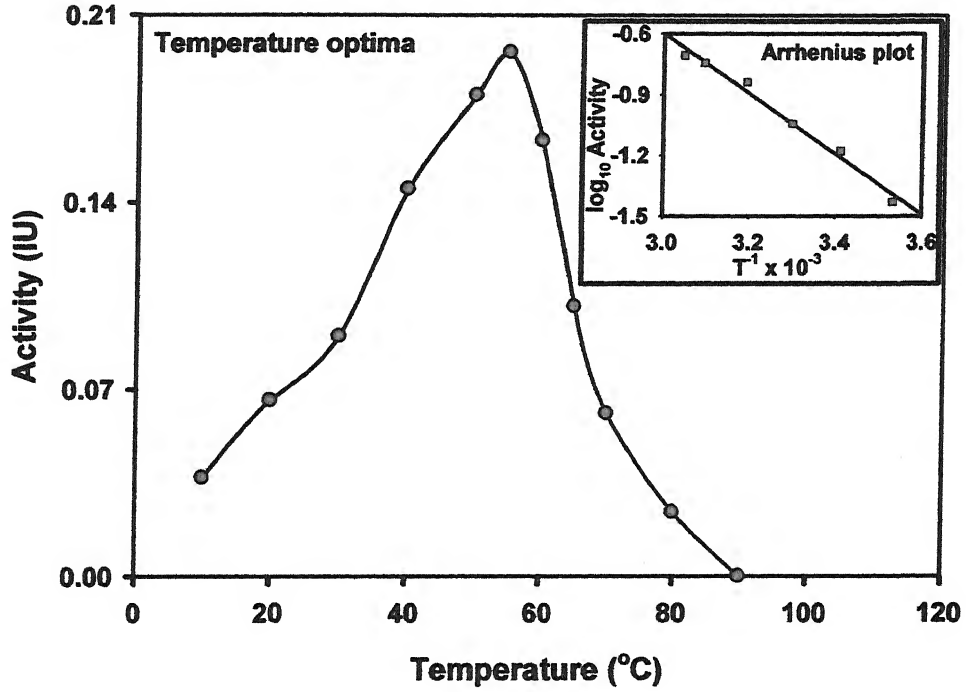


Figure 21: Temperature optima and thermotropic behaviour of *Andrographis* β-glucosidase.

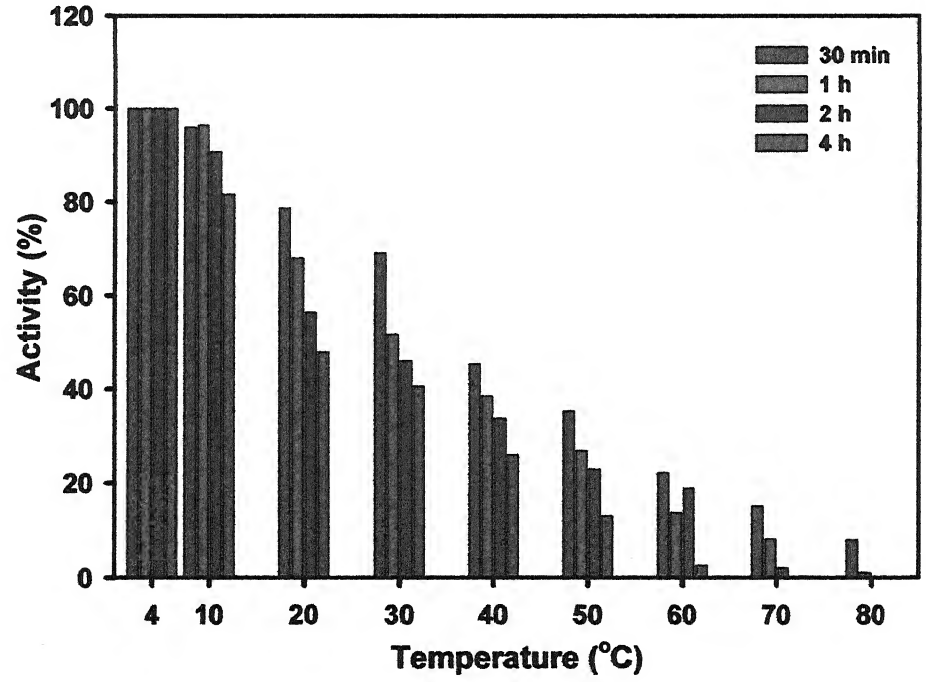


Figure 22: Temperature stability profile of *Andrographis* β-glucosidase.

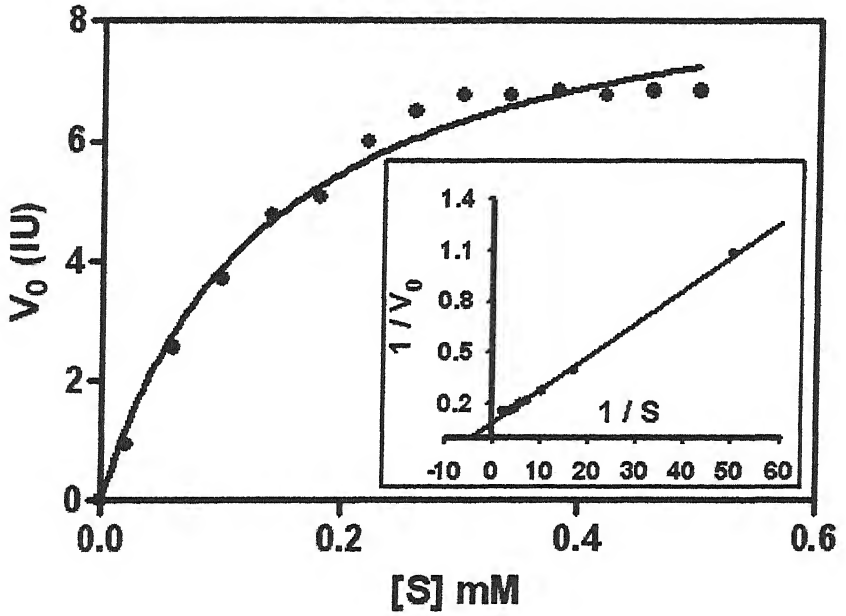


Figure 23: Substrate (*p*NPG) saturation kinetics of *Andrographis paniculata* β-glucosidase.

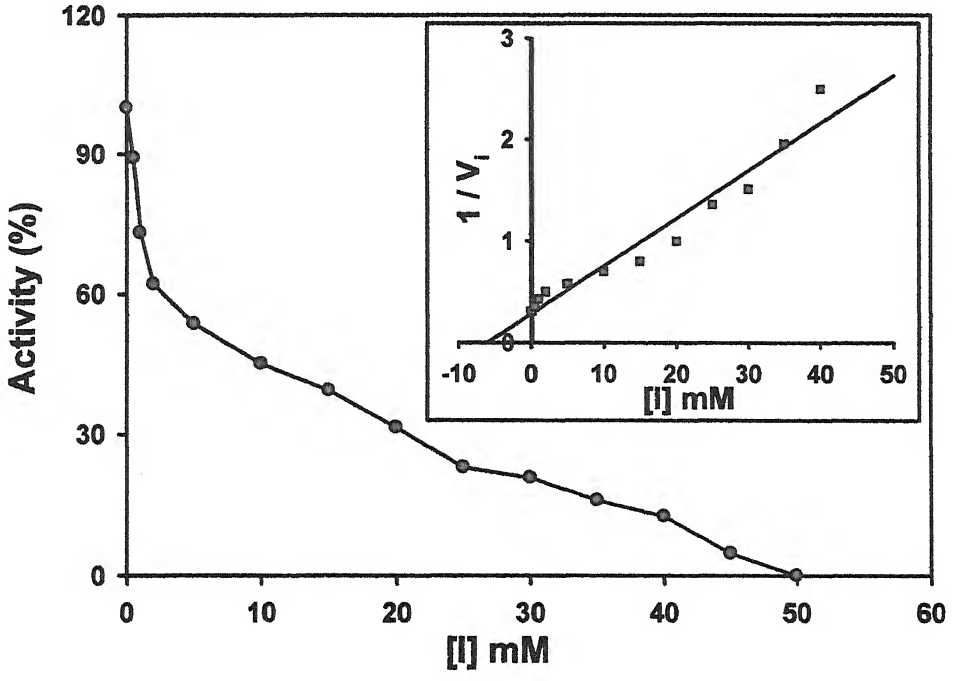


Figure 24: Effect of D-Gluconic acid lactone on *Andrographis paniculata* β-glucosidase.

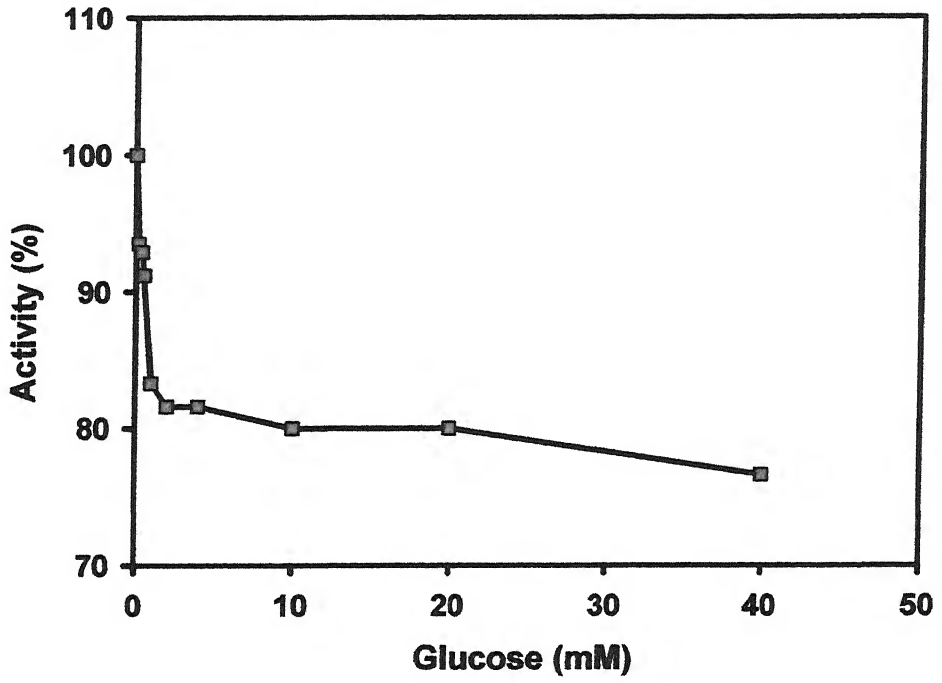


Figure 25: Effect of glucose on *Andrograhis paniculata* β-glucosidase.

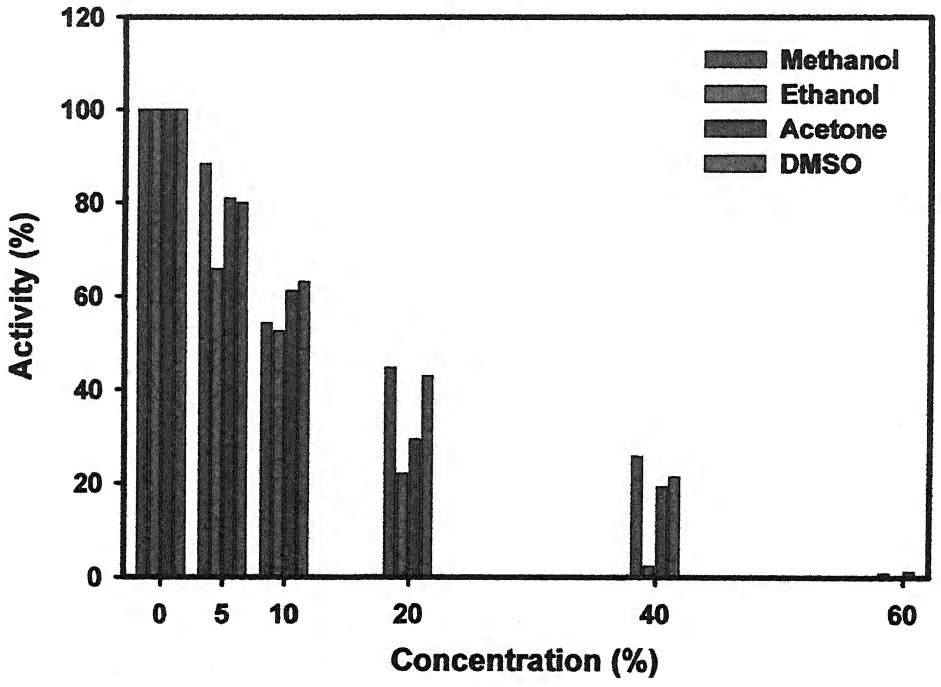


Figure 26: Effect of organic solvents on *Andrographis paniculata* β-glucosidase.

4.2.11. Effect of cations on β-glucosidase activity

A range of mono-, di- and tri-valent metal ions was tested of their effect on the enzyme activity. The effect of, K^+, Na^+, Zn^{2+}, Mg^{2+}, and Cu^{2+} on the catalytic activity was none at 1 mM concentration and negligible at 10 mM. But Li^+ showed moderate inhibition of 10% and 25% at 1.0 mM and 10 mM concentrations, respectively. Ag^+, the well know inhibitory metal ion for β-glucosidases strongly inhibited *Andrographis* enzyme also- 75% and 94% at 1 mM and 10 mM concentrations, respectively. Among divalent cations, Mn^{2+} and Ca^{2+} were moderately inhibitory even at high concentration (10 mM) (Table 8). The only metal ion activator was Fe^{2+} which activated the enzyme slightly at 1.0 mM but markedly (>1.4 fold) at 10 mM. Contrarily, Fe^{3+} was inhibitory. Hg^{2+} was also inhibitory with 43 and 83% inhibition observed at 1.0 and 10 mM, respectively. Ag^+ was found to be strong inhibitor of the *Andrographi paniculata* enzyme. Thiol directed reagent, iodoacetate, had little effect on the enzyme (20 and 27 % inhibition at 1 mM and 10 mM concentrations, respectively) (Table 8).

The thiol directed reagent iodoacetate showed very slight inhibitory effects on the enzyme activity- 20 and 23% inhibition at 1 and 10 mM, respectively (Table 8).

4.2.12. Effect of organic solvents

Effect of miscible organic solvents (ethanol, methanol, acetone and dimethyl sulphoxide) on β-glucosidase activity was observed at their 10 to 50% (v/v) concentration. Dimethyl sulphoxide (DMSO) reduced the catalytic activity by about 37% and 90% at 10% and 50% (v/v) concentrations (Fig. 26). A similar pattern of solvent effect on the enzyme activity was observed with methanol, acetone and ethanol with slight variation in quantitative values of inhibition. In a relative account, methanol was the least effective and ethanol was the most (Fig. 26).

Table 8: Effect of metal ions on activity of *Andrographis paniculata* β-glucosidase.

Effector	Activity (% of control)	
	1 mM	10 mM
Li^+	90.3	75.0
K^+	96.9	94.3
Na^+	97.9	78.5
Ag^+	25.5	6.6
Hg^{2+}	57.1	17.3
Ni^{2+}	102.0	98.4
Mn^{2+}	90.8	80.1
Mg^{2+}	96.9	95.9
Ca^{2+}	80.1	71.9
Cu^{2+}	98.4	81.6
Zn^{2+}	104.0	106.6
Fe^{2+}	107.1	141.8
Fe^{3+}	96.4	71.4
Iodoacetate	80.1	73.4

4.2.13. Transglycosylation

Transglucosylating activity was noted with *Andrographis* β-glucosidase under lower aqueous micromilieu generated by the presence of DMSO (10%). The transglycosylation assay mixture contained different aglycone acceptors like methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, decanol, geraniol, linalool, menthol, quercetin, and withaferin-A and used cellobiose or *p*NPG as the glucosyl donor (Fig. 27). TLC analyses of the catalytic reaction products were carried out and the chromatograms were compared with appropriate controls. Glucoside formation was observed (R_f 0.27) only with geraniol as the aglycone acceptor and *p*NPG as the glycosyl donor (Fig. 28).

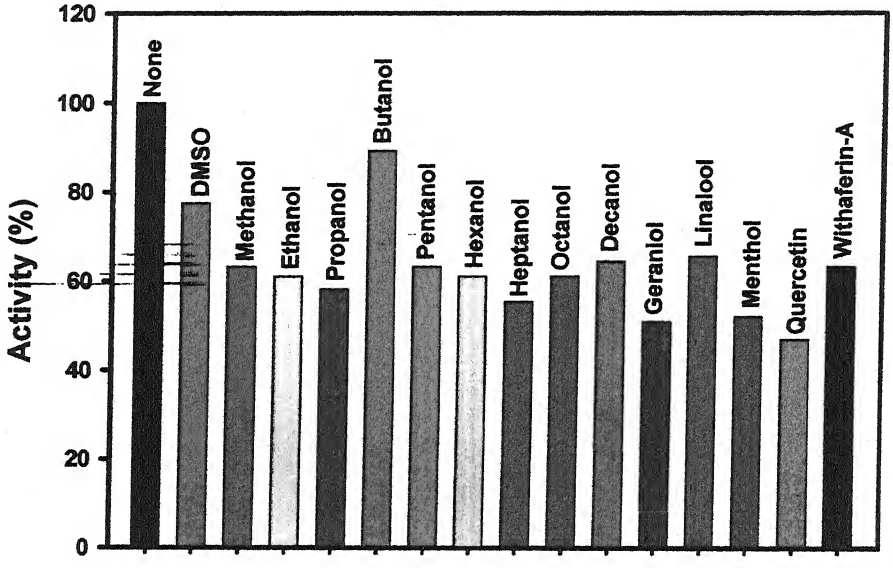


Figure 27: Effect of glucosyl donors on *Andrographis paniculata* β-glucosidase.

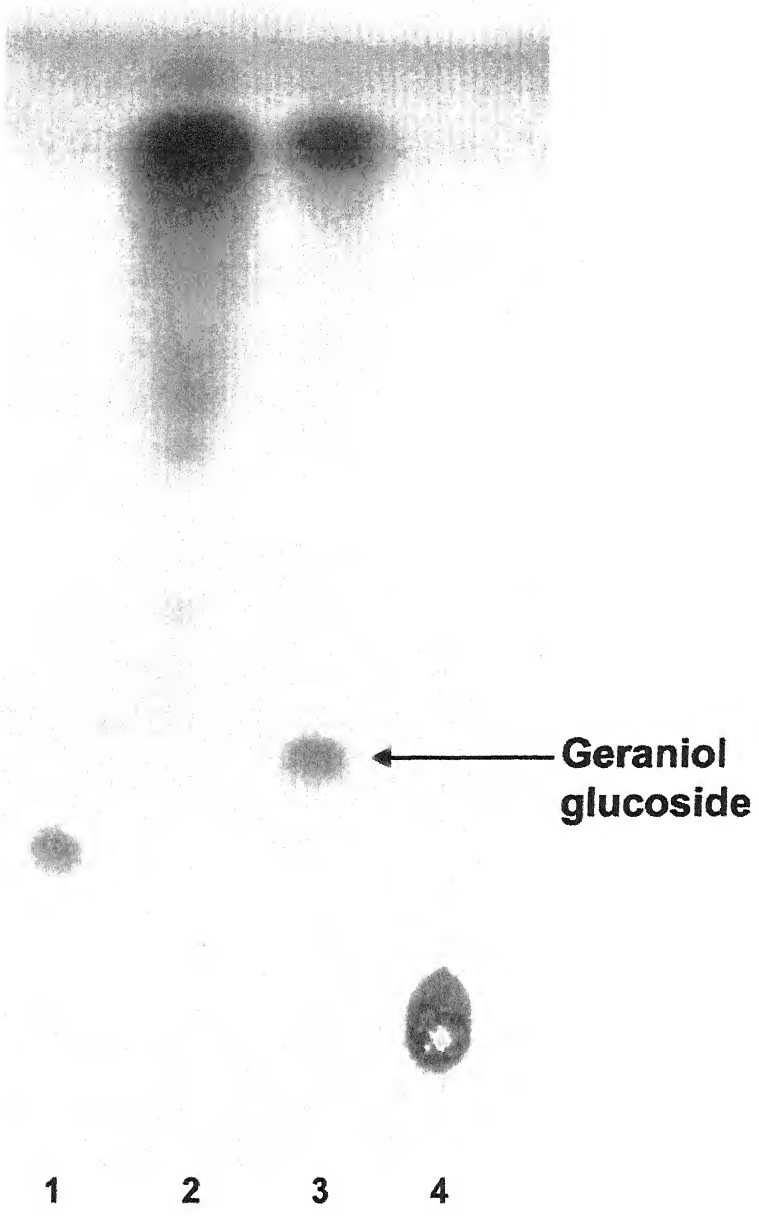


Figure 28: TLC analysis of transglucosylation reaction catalyzed by *Andrographis paniculata* β-glucosidase. Lane: 1, *p*NPG; 2, geraniol standard; 3, geraniol reaction with *p*NPG; 4, enzyme extract fractionated with ethyl acetate.

4.3. *Silybum marianum* petal β-glucosidase

In this set of the investigation, β-glucosidase from *Silybum marianum* (milk thistle) flower petals has been purified through a combination of differential precipitation and column chromatography. The purified enzyme has been characterized with respect to its physical and kinetic properties including effectors of the castalytic activity. The final enzyme preparation was composed of two polypeptides, one of which was established to be a contaminant fucosyltransferase that was characterized bioinformatically for the limited motifs of high sequence conservation.

4.3.1. Optimized enzyme extraction for purification

The enzyme isolation optimizations for *Silybum* β-glucosidase were performed to obtain high specific activity enrichment to facilitate minimized co-extraction of other proteins and thereby aid the purification. The enzyme extracted from the petals in extraction buffer (100 mM citrate-phosphate) pH 5.0 showed highest β-glucosidase activity while at that at pH 3.0 had little (7.3%) activity (Table 9). Total protein content was obtained highest at pH 6.0 with almost equal activity than pH 5.0 therefore slightly less specific activity was recorded at pH 6.0 and in turn, highest specific was obtained at extraction buffer pH 5.0 (Table 9).

Table 9: Enzyme isolation optimization for *Silybum marianum* β-glucosidase.

Extraction buffer pH	Protein (mg / gm f.wt.t.)	Activity (IU / gm f.wt.t.)	Specific Activity (IU / mg)
3.0	1.660	0.087	0.052
4.0	2.760	0.262	0.095
5.0	4.480	1.190	0.265
6.0	5.170	1.160	0.224

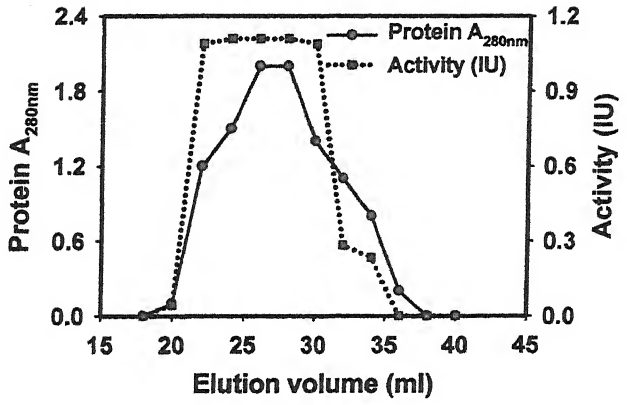
4.3.2. *Silybum marianum* petal β-glucosidase purification

The differential ammonium sulfate (10-40%) saturation precipitation of the crude homogenate resulted in about 3 fold purification with 27% recovery (Fig. 29A; Table 10). A very high enrichment of the enzyme activity (44 fold with 15% recovery) was achieved by the anion exchange (Q-Sepharose) chromatography (Fig. 29B). Final purification of the order of 1181 fold (with 7.5% recovery) was achieved through cation exchange (S-Sepharose) chromatography (Fig. 29C; Table 10). The enzyme preparation on SDS-PAGE showed two bands after Coomassie as well as silver stain (Fig. 30). Both protein bands were sequence on MS/MS MALDI-Tof and the lower band was ascertained to be β-glucosidase whilst the upper was found to be a contaminant fucosyltransferase. The purified enzyme preparation was characterized physico-kinetically for the comparative account of the catalytic attributes.

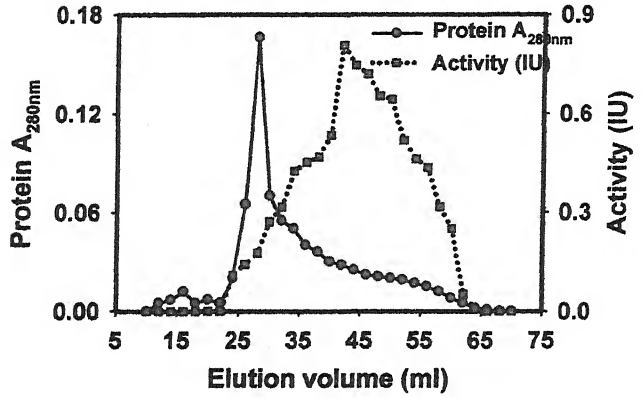
Table 10: Purification profile of *Silybum marianum* petal β-glucosidase.

Purification Step	Volume (ml)	Total Activity (IU)	Total Protein (mg)	Specific Activity	Purifi-cation Fold	Recovery (%)
Crude Homogenate	40	40.0	88.32	0.452	1	100
Sephacryl S-200-HR	10	11.0	8.74	1.258	2.7	27.5
Q-Sepharose	20	6.0	0.300	20.0	44.2	15.0
S-Sepharose	4	3.0	0.0056	535.71	1185.1	7.5

A



B



C

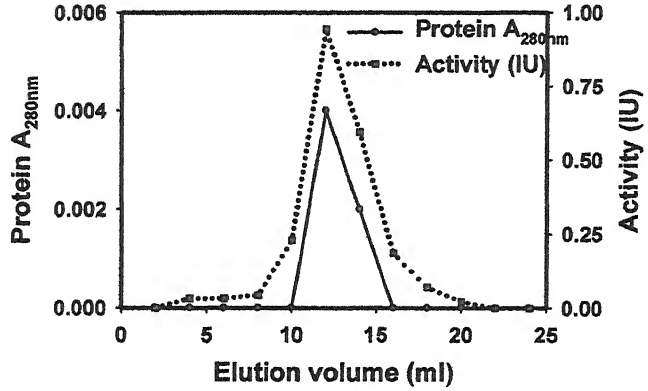


Figure 29: Protein and activity profile of β-glucosidase at different chromatographic purification steps in *Silybum marianum*. (A) Sephacryl S-200HR, (B) Q-Sepharose, (C) Q-Sepharose.

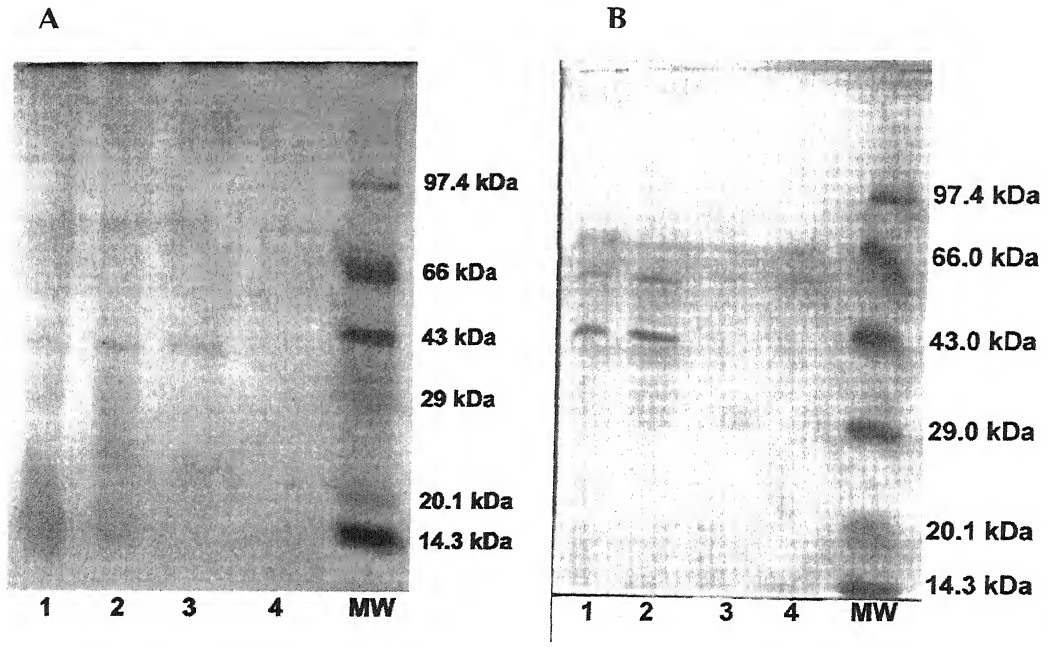


Figure 30: SDS-PAGE (A, silver stained; B, Coomassie stained) for *Silybum marianum* petal β-glucosidase at different stages of purification. Lanes: 1, crude homogenate; 2, Sephacry-200 HR; 3, Q-Sepharose; 4, S-Sepharose; MW, standard molecular weight marker (Banglore Genie, India)

4.3.3. Native molecular weight and subunit composition

The native molecular weight for *Silybum* enzyme was determined by size exclusion chromatography through a Sephcryl S-200-HR column. A plot of $\log_{10}$ MW *versus* portioning coefficient (K_{av}) (Fig. 31A) led to its native molecular weight estimate of 135 kDa. A calibration plot was drawn between $\log_{10}$ molecular weight *versus* migration distance of standard molecular weight proteins from SDS-PAGE (Fig. 31B). That plot was used to compute subunit (lower band) molecular weight of *Silybum* β-glucosidase as 67.5 kDa which suggested that the enzyme was a homodimeric (Fig. 30). On gel upper (fucosyl transferase) and lower (β-glucosidase) polypeptides constituted 60% and 40%, respectively of the protein in final enzyme preparation.

4.3.4. pH optima and pH stability

The plot of pH *versus* catalytic activity of *Silybum* β-glucosidase was typical bell-shaped that gave a pH optimum value of 5.5 (citrate-phosphate buffer) (Fig. 32).

pH stability of the enzyme stability was observed by incubating the preparation at different pH (100 mM citrate-phosphate pH 3.0, 4.0, 5.0, 6.0 and 100 mM Tris-buffer pH 7.0, 8.0, 9.0, and 10.0) for different durations (30 min, 1 h, 2 h, and 4 h) (Fig. 33). The enzyme observed to have maximal stability at pH 6.0 with better stability towards acidic pH range.

4.3.5. Thermostability and thermotropic behaviour

The themperature optima of *Silybum* β-glucosidase was estimated to be 40 °C from the plot of assay temperature *versus* catalytivc activity (Fig. 34). An Arrhenius plot was drawn between $\log_{10}$ activity *versus* inverse of absolute temperature (1/T) (Fig. 34*inset*) to compute the energy of activation (E_a) of the enzyme. The plot was a single straight line and gave energy of activation estimated of 8.26 $kCal.Mol^{-1}$.

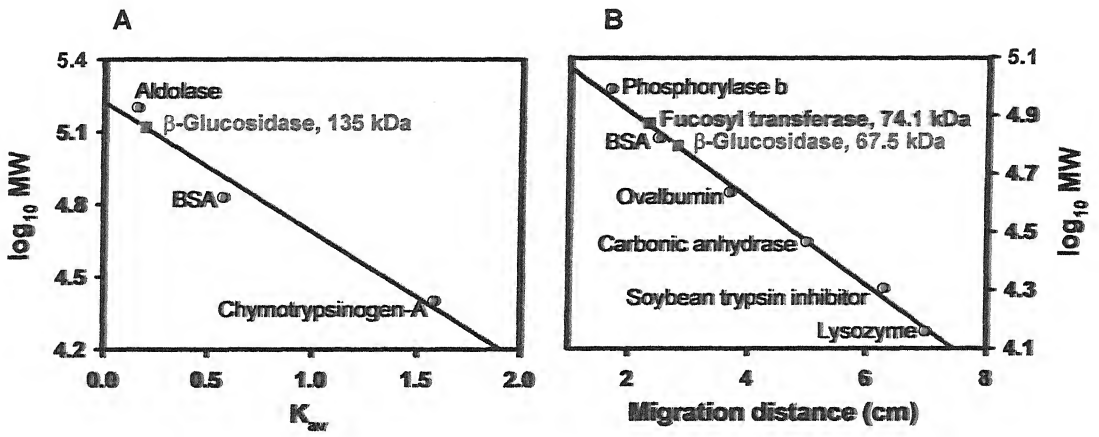


Figure 31: (A) Native MW calibration plot from Sephacryl S-200-HR, (B) Subunit MW calibration plot from SDS-PAGE.

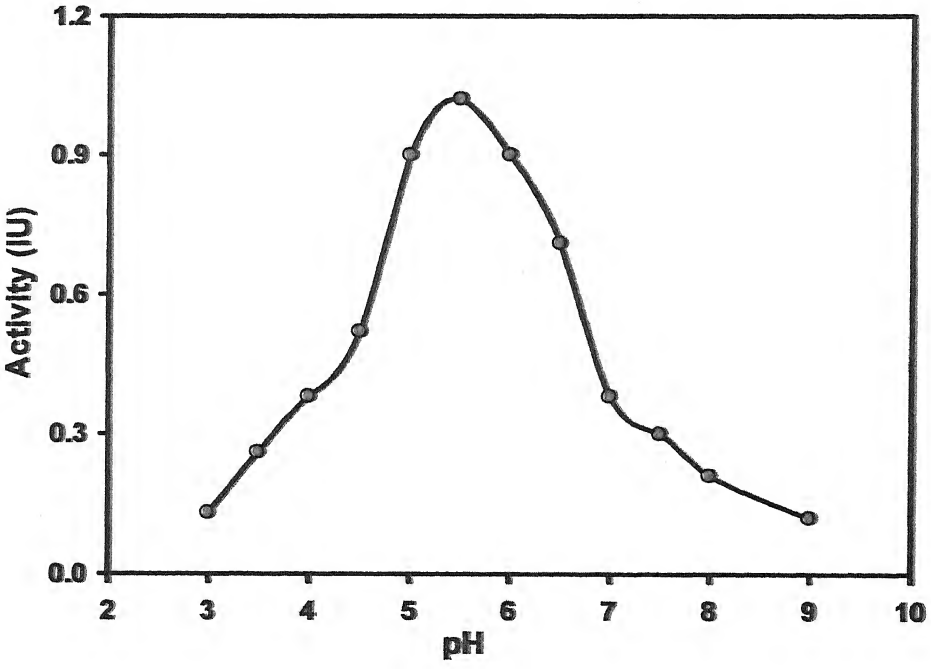


Figure 32: pH optima of *Silybum marianum* β-glucosidase

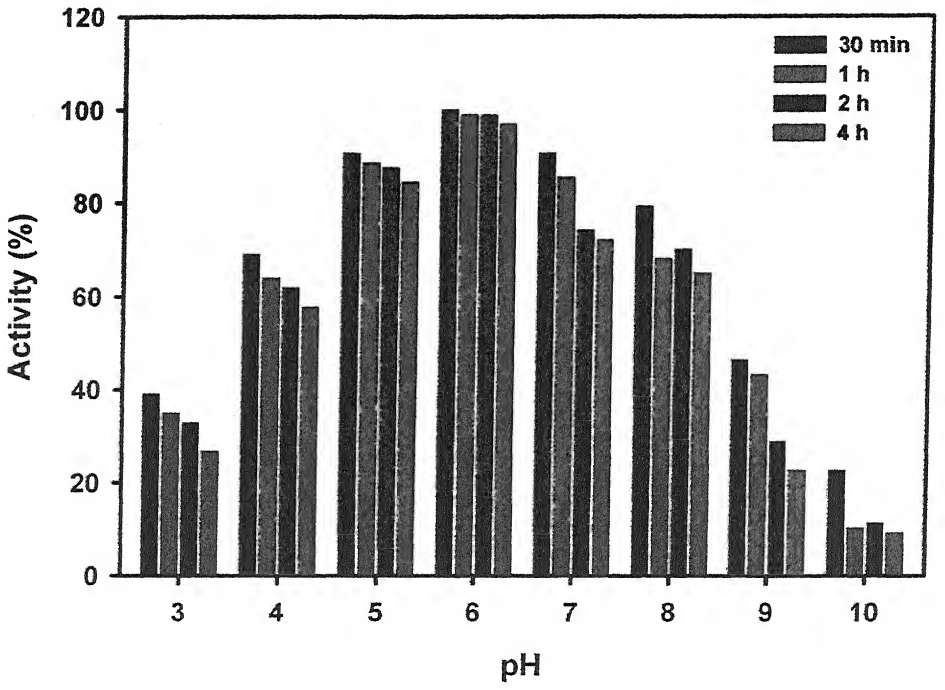


Figure 33: pH stability profile of *Silybum marianum* β-glucosidase.

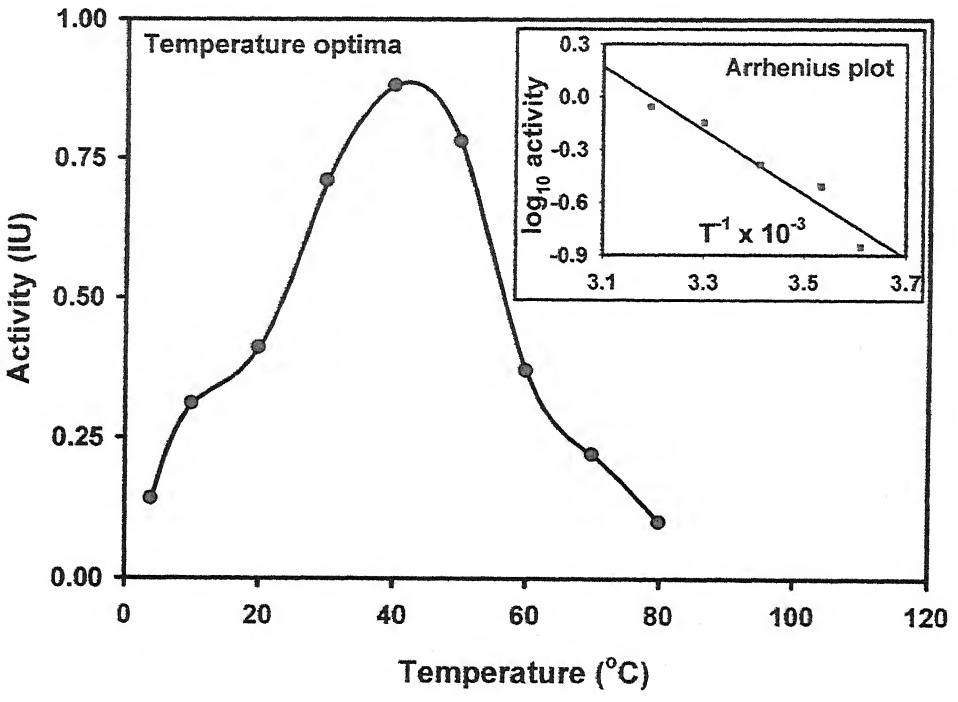


Figure 34: Temperature optima and thermotropic behaviour of *Silybum marianum* β-glucosidase.

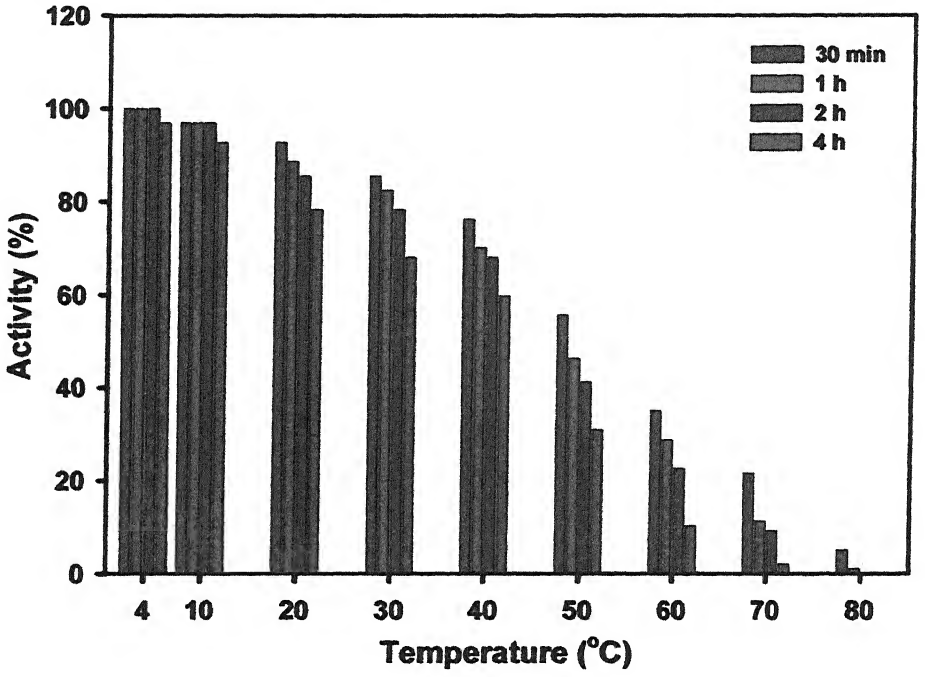


Figure 35: Temperature stability profile of *Silybum marianum* β-glucosidase.

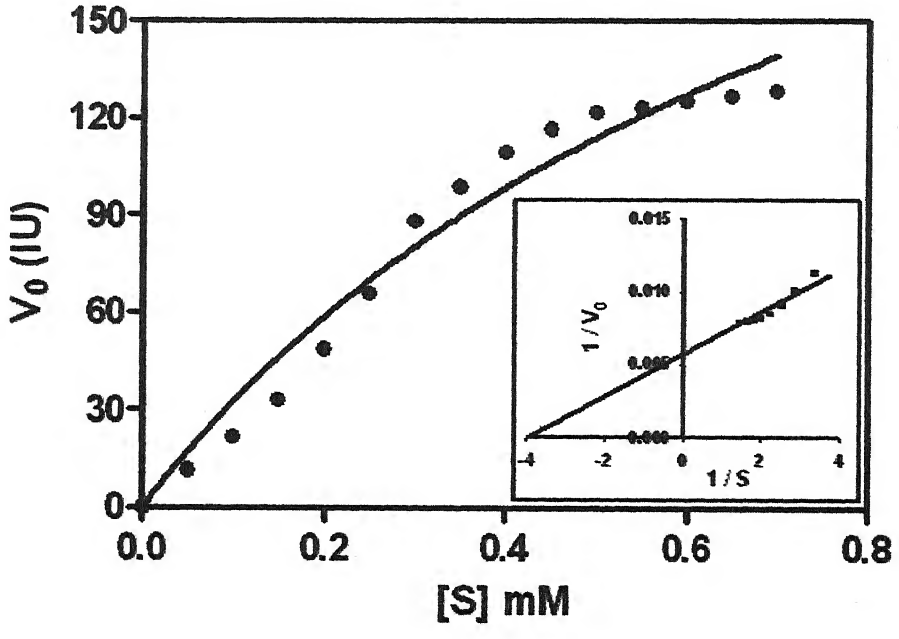


Figure 36: Substrate saturation kinetics of *Silybum marianum* β-glucosidase.

Thermostability of the enzyme was analyzed by monitoring the residual catalytic activity of the enzyme after its incubation at different temperatures. The profile showed that the enzyme had best stability in the temperature range 4-10 °C (Fig. 35).

4.3.6. Substrate saturation kinetics

A normal hyperbolic type of substrate saturation curve was displayed by the enzyme for *p*-nitrophenyl-β-D-glycopyranoside suggesting it to follow typical Michaelis-Menten kinetics (Fig. 36). The values for K_m and V_{max} were estimated to be 0.25 mM and 130.0 IU, respectively. Enzyme catalytic efficiency (K_{cat}/K_m), the more rational kinetic parameters of ranking catalytic performance was computed to be 619,000 $M^{-1}.s^{-1}$.

4.3.7. Substrate specificity

The substrate specificity of enzyme was assessed using various substrates analogues with different types of linkages (β or α), different glyco-moieties and alternate position of the substituent group (-NO_2) on the aglycone moiety (Table 11). These included several β-linked sugars conjugates and aglycone substituent group position variants (Table 11).

The enzyme exhibited highest deglycosylating actvity with β-*p*NPGlu but >50% of this activity was also recorded with β-*p*NPFuc. The enzyme also showed significant (28%) with *ortho* substituted nitrophenyl (aglycone) substrate β-*o*NPGlu. Also noticeable activity was noted with β-*p*NPGal and α-*p*NPGlu (Table 11). These results suggested that the enzyme was not absolutely substrate specific and showed flexibility, though limited, to function with respect to linkage for β-type and galacto-conjugates.

Table 11: Substrate specificity of *Silybum marianum* β-glucosidase.

S. No.	Substrate	Activity (%)
1	*p*-nitrophenyl-β-D-glucopyranoside (β-*p*NPGlu)	100
2	*p*-nitrophenyl-α-D-glucopyranoside (α-*p*NPGlu)	5.4
3	*o*-nitrophenyl-β-D-glucopyranoside (β-*o*NPGlu)	28.3
4	*p*-nitrophenyl-β-D-galactopyranoside (β-*p*NPGal)	14.1
5	*p*-nitrophenyl-α-D-galactopyranoside (α-*p*NPGal)	0.9
6	*o*-nitrophenyl-β-D-galactopyranoside (β-*o*NPGal)	0.4
7	*p*-nitrophenyl-β-D-mannopyranoside (β-*p*NPMan)	0.2
8	*p*-nitrophenyl-β-D-xylopyranoside (β-*p*NPXyl)	0.1
9	*p*-nitrophenyl-β-D-fucopyranoside (β-*p*NPFuc)	54.3
10	*p*-nitrophenyl-β-D-lactopyranoside (β-*p*NPLac)	0.6

4.3.8. Gluconolactone Inhibition kinetics

Silybum marianum enzyme showed very low sensitive to inhibition with D-glucono-1,5-lactone as complete inhibition was notice at its very high (50 mM) concentration (Fig. 37). The inhibition pattern showed that at initial gluconolactone (1-5 mM) concentration the activity is steeply inhibited thereafter a saturation kind of situation is observed and hence in the range of 30-60 mM gluconolactone concentration only 12% loss in activity (87-99%) is recorded. From the analysis of the inhibition kinetics the inhibition constant (K_i) for the enzyme was estimated to be 3.8 mM (Fig. 37*inset*).

4.3.9. Effect of glucose

Glucose was found to have no effect on *Silybum marianum* β-glucosidase activity when tested in the concentration range of 2 to 40 mM in the assay mixture.

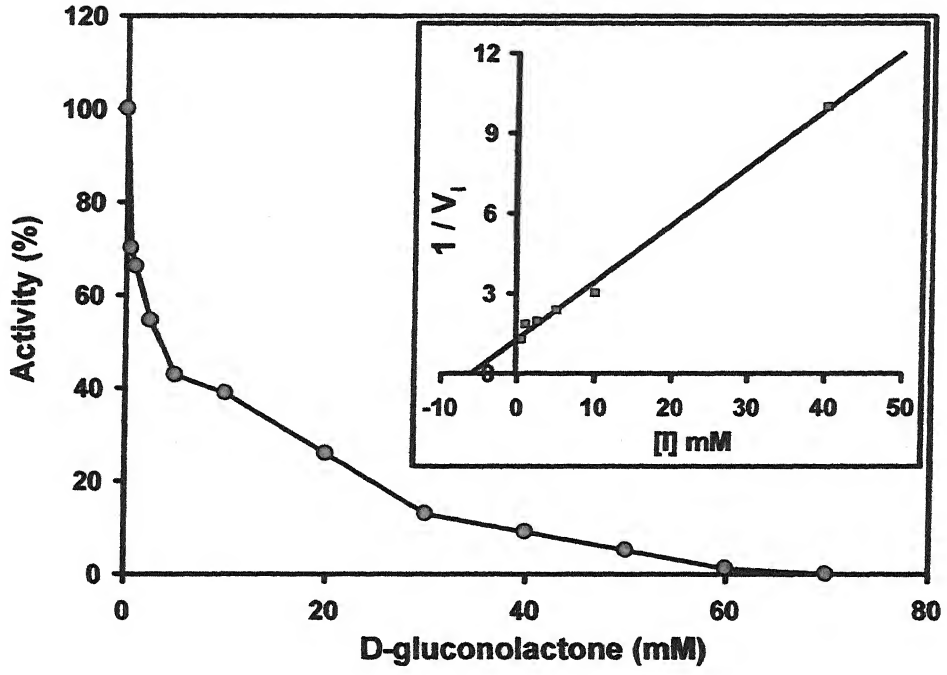


Figure 37: D-gluconolactone inhibition kinetics of *Silybum marianum* β-glucosidase.

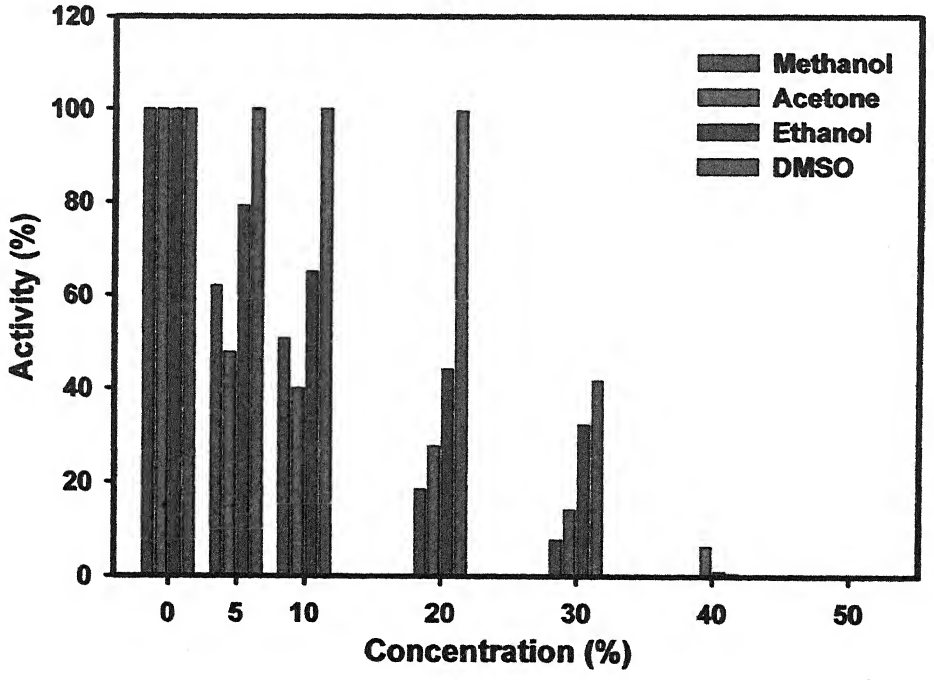


Figure 38: Effect of organic solvents on *Silybum marianum* β-glucosidase.

Table 12: Effect of cations and chelating agents on *Silybum marianum* β-glucosidase.

Effector	Activity (% of control)		
	1 mM	2.5 mM	5 mM
Li^+	105.7	104.9	100.3
K^+	98.3	79.0	72.1
Na^+	101.6	97.5	92.2
Ag^+	6.2	4.0	0.8
Hg^{2+}	8.1	0.8	0.1
Ni^{2+}	126.2	110.6	100
Zn^{2+}	94.2	94.2	94.4
Co^{2+}	91.8	88.0	59.0
Mn^{2+}	96.0	81.9	63.7
Cu^{2+}	98.6	80.3	71.3
$Cd^{2+}{}_2$	98.3	89.8	73.7
Mg^{2+}	101.6	102.4	102.4
Fe^{2+}	100	121.3	313.1
Fe^{3+}	96.7	100.8	131.9
EDTA	99.5	99.3	98.3
EGTA	99.1	97.5	95.0

4.4.10. Effect of cations and chelating agents

Effect of three different concentrations (1.0, 2.5, and 5.0 mM) of metal ions tested for their effect on *Silybum* β-glucosidase included monovalent cations (Li^+, K^+, Na^+, and Ag^+), divalent cations (Hg^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}, Mg^{2+}, and Fe^{2+}) and the only trivalent cation (Fe^{3+}). Li^+, K^+, and Na^+ had no marked effect on enzyme activity while Ag^+ was strongly inhibitory (94% 1 mM concentration).

Likewise, Hg^{2+} amongst divalent cations was highly inhibitory (92% at 1 mM). Other divalent cations had no or little effect on enzyme activity at physiological concentrations (1 to 2.5 mM). Fe^{2+} and Fe^{3+} were found to be activator for the enzyme activity, though the pattern lacked linear response to the concentration (Table 12).

4.4.11. Effect of organic solvents

The effect of miscible organic solvents at varying concentrations (5 to 50%, v/v) using ethanol, methanol, acetone and dimethyl sulphoxide was tested on β-glucosidase activity (Fig. 38). Among all three tested solvents, methanol was found to have highly diminishing effect on enzyme activity which inhibited about 40% activity at 5 % and more than 92% activity at 30% concentration. Ethanol and acetone also have similar activity decreasing fashion of but with a low tendency while DMSO has very different behavior on enzyme activity. DMSO had no effect on enzyme activity up to 20% concentration and a sudden loss of about 60% activity is recorded at 30% concentration whereas 40% concentration caused almost complete inhibition of enzyme activity (Fig. 38).

4.4.12. Transglycosylation

To explore the transglucosylating catalytic feasibility of *Silybum marianum* petal β-glucosidase a lower aqueous micromilieu was generated using 10% DMSO. The standard assay mixture for transglycosylation reaction contained different aglycone acceptors like methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, decanol, geraniol, linalool, menthol, quercetin, withaferin-A, and ursolic acid (Fig. 39). Glycosyl donors used were cellobiose or *p*NPG and the reaction product analysis was performed on thin layer chromatographic with appropriate controls.

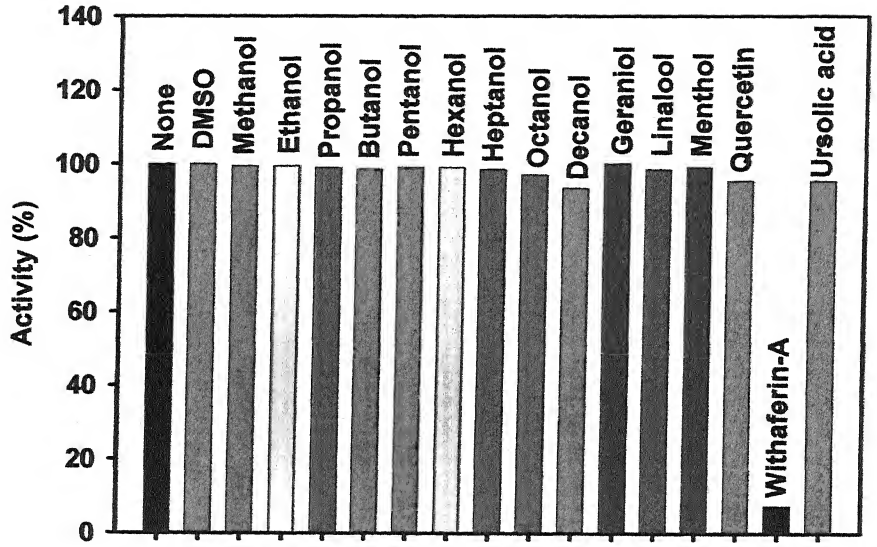


Figure 39: Effect of glycosyl donors on *Silybum marianum* β-glucosidase activity.

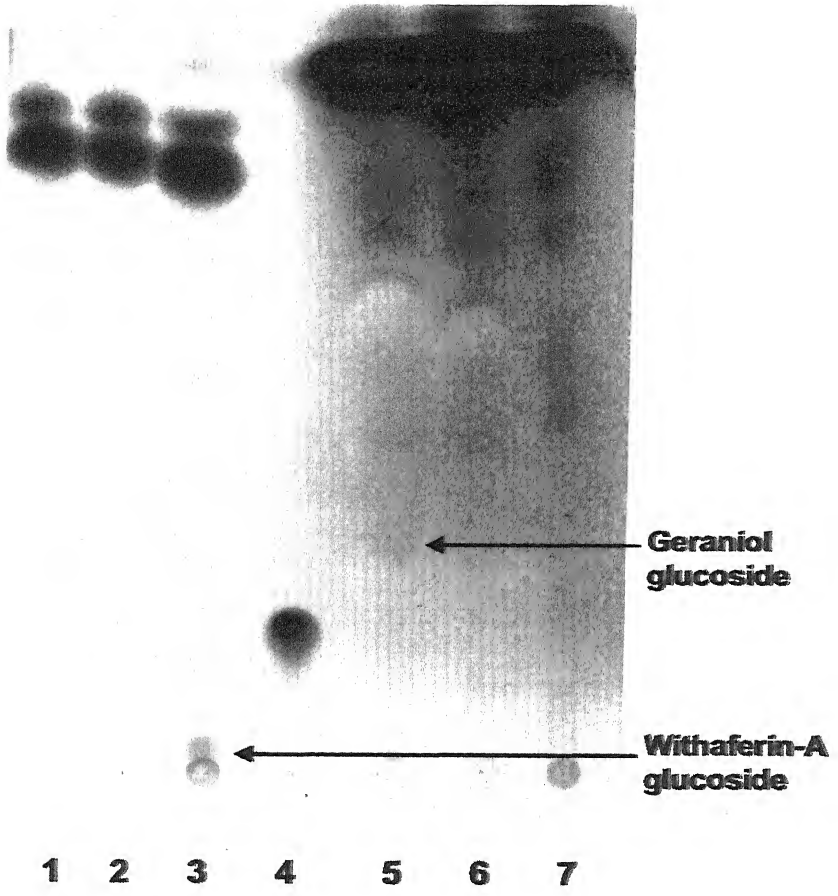


Figure 40: TLC analysis of transglucosylation reaction catalyzed by *Silybum marianum* petal β-glucosidase. Lane: 1, withaferin-A standard; 2, withaferin-A reaction with cellobiose; 3, withaferin-A reaction with *p*NPG; 4, *p*NPG standard; 5, geraniol reaction with *p*NPG; 6, geraniol reaction with cellobiose; 7, geraniol standard.

The formation of glucosides was observed with two aglycone moieties using *p*-nitrophenol β-D-glucopyranoside as glycosyl donor and the aglycone acceptors were geraniol and withaferin-A (Fig. 40). The transglucosylated product formed were identified as geraniol glucoside (R_f 0.28 i.e. slightly above *p*-NPG) and withaferin-A glucoside (R_f 0.14 i.e. slightly below *p*-NPG). The transglycosylation reaction using cellobiose did not give any product formation with any of the substrates suggesting that cellobiose could not serve as a glucosyl donor for *Silybum* β-glucosidase.

4.5. Proteomic analysis of *Silybum marianum* petal β-glucosidase

The lower (67.5 kDa) polypeptide from SDS-PAGE was excised from the Coomassie stained gels and subjected to proteomic analysis (limited peptide sequence fingerprinting) through MALDI-TOF-TOF. The MS and MS/MS spectrum acquired on MALDI-TOF/TOF Mass Spectrometer.

The two most abundant *de novo* sequences were subjected to BLAST analysis that gave a very high matching with *Stigmatella aurantiaca* 1,4-β-D-glucan glucohydrolase D (STI), *Bacillus thuringiensis* 6-aminohexanoate-dimer hydrolase (BCT), *Nitrosococcus oceani* ATCC 19707 glycoside hydrolase, family 57 (NIT), and *Bacillus halodurans* β-glucosidase (BAH) (Fig. 41). The sequence homology with glycosides corroborated that the peptide fragment generated in final purification of *Silybum marianum* petal enzyme was glucosidase.

4.6. Identification of a fucosyl transferase from *Silybum marianum* petals

The SDS-PAGE gel for purification of *Silybum* petal enzyme showed had, besides the 67.5 kDa β-glucosdidase, a contaminating 74.1 kDa polypeptide. The peptide digestion and fragment analysis of the 74.1 kDa polypeptide on MALDI-

TOF was carried out to have the resultant *de novo* fragment masses (sequences). The two most predominant fragment sequences were BLAST searched for the sequence matching. After sequence matching and multiple sequence alignment, the sequence was found to pertain to fucosyltransferase. The BLAST analysis of sequence provided 100% sequence homology of two peptides with the sequences of *Arabidopsis thaliana* xyloglucan fucosyltransferase (Accession Q9SJP6) and *Arabidopsis thaliana* putative fucosyltransferase 10 (Accession Q9SJP6). A hybrid variety of *Populus* (*Populus tremula* x *Populus alba*) enzyme named α-1,2-fucosyltransferase (PpFUT) (Accession ABS70454) also had good sequence match with that of *Silybum* enzyme. Similarly, the enzyme has high homology to *Oryza sativa* putative xyloglucan fucosyltransferase (OsFUT1) (Accession BAD17112), *Oryza sativa* Japonica cultivar putative xyloglucan fucosyltransferase (OsFUT1) (Accession BAD17106), and *Oryza sativa* Japonica cultivar putative galactoside 2-α-L-fucosyltransferase (OsFUT1) (Accession BAD17223), respectively (Fig. 42).

```
SaGS     766 -VTPNNEVT- 773     NoGS     244 -NTSEESNF- 251
BtGS     394 -ATPSNEIH- 401     BhGS     384 -KRSMESGV- 391
SmGS1        -VTPSNEVH-        SmGS2        -KRSEESNF-
                :**:**:                    ..* ** .
```

Figure 41: Protein sequence analysis. SaGS, *Stigmatella aurantiaca* 1,4-β-D-glucan glucohydrolase D (Nierman, 2006); BtGS, *Bacillus thuringiensis* 6-aminohexanoate-dimer hydrolase (Han *et al.*, 2006); NoGS, *Nitrosococcus oceani* ATCC 19707 Glycoside hydrolase, family 57 (Klotz *et al.*, 2006); BhGS, *Bacillus halodurans* β-glucosidase (Takami *et al.*, 2000); SmGS, *Silybum marianum* β-glucosidase fragments 1 and 2. Indications- : conservative change; . semi conservative change; * complete match.

```
AtFUT1   349 YYEAYLSHADEK 360   506 ITPDPSCGR 514
AtFUT2   302 YYDAHLSNADER 313   458 ITPNPPCVR 466
PpFUT    355 FYRAYLSKANET 366   513 ITPDPPCVQ 521
OsFUT1   382 YYRAYLATARQL 393   549 AVPDPPCGR 617
OsFUT2   363 YYEAYLARVDEK 374   521 ERSEVACVR 529
OsFUT3   165 FYEAYLARADEL 176   329 ARAEPACVR 337
SmFUT        YYEAYLSHADEK          ITPDPSCGR
               :*.*:*:.::            ...::.*.:
```

Figure 42: Sequence alignment of fucosyltransferases protein sequences. AtFUT1, *Arabidopsis thaliana* xyloglucan fucosyltransferase (Perrin *et al.*, 1999); AtFUT2, *Arabidopsis thaliana* putative fucosyltransferase 10 (Sarria *et al.*, 2001); PpFUT, *Populus tremula* x *Populus alba* α-1,2-fucosyltransferase (Costa *et al.*, 2007); OsFUT1, *Oryza sativa* putative xyloglucan fucosyltransferase (Sasaki *et al.*, 2002); OsFUT2, *Oryza sativa* Japonica cultivar putative xyloglucan fucosyltransferase (Yu *et al.*, 2005); OsFUT3, *Oryza sativa* Japonica cultivar putative galactoside 2-α-L-fucosyltransferase (Yu *et al.*, 2005); SmFUT, *Silybum marianum* fucosyl transferase fragments. Indications- : conservative change; . semi conservative change; * complete match.

5.

DISCUSSION

This study on β-glucosidase from selected medicinal plants explored multi facets of enzyme's properties-catalytic kinetics, physical properties including size and sub-unit composition and functional properties including substrate specificity and activity effectors. The properties have been compared among themselves as well as those from other plant, animal and microbial sources. The knowledge on the library of the enzymes could be a useful resource for structure functional understanding of the catalysis, considering that enzymes with novel functions and properties can be obtained either by searching the largely unknown natural species or by improving upon the currently known ones through mutagenesis at putative residues of significance in catalysis. Besides, the diversity of their catalytic properties not only underpins their variable metabolic role for the species/tissue but also invokes their potential application in biotransformation.

5.1. Size and subunit composition

The native molecular weight β-glucosidases purified from leaf of *Withania somnifera* and *Andrographis paniculata* were 50 and 60 kDa, respectively whereas size of the enzyme from *Silybum marianum* petal was twice larger (135 kDa). The subunit molecular weights were 50 and 60 kDa for *Withania somnifera* and *Andrographis panicuata* β-glucosidases, respectively implying both of them to be monomeric in nature. The enzyme from *Silybum marianum* petal was composed of two polypetides of the same size (67.5 kDa) i.e. it was homodimer. Coincidently, the final purified enzyme preparation from *Silybum* was not at homogeneity as on SDS-PAGE gel, it had two bands - the larger (74.1 kDa) and smaller (67.5 kDa) that grossly constituted 60% and 40% of the protein in the preparation, respectively. However, the homodimeric organization of *Silybum* needed to be ascertained by advanced proteomic investigations.

Table 1: Molecular weight of β-glucosidases from different sources.

Source	Native Molecular Weight (kDa)	Subunit Molecular Weight (kDa)	Reference
		Plants	
Withania somnifera	50 kDa	50 kDa (monomer)	Present Work
Andrographis paniculata	60 kDa	60 kDa (monomer)	Present Work
Silybum marianum	135 kDa	67.5 kDa (homodimer)	Present Work
Strychnos mellodora	~200 kDa	~80 kDa (homodimer)	Brandt *et al.*, 2000
Dalbergia cochinchinensis	330 kDa	66 kDa (homopentamer)	Srisomsap *et al.*, 1996
Catharanthus roseus	63 kDa	63 kDa (monomer)	Luijendijk *et al.*, 1998
Vigna radiate	nd	95 kDa	Zeng and Elbein, 1998
Oryza sativa	nd	56 kDa	Akiyama *et al.*, 1998
Malus domestica	120 kDa	60 kDa (homodimer)	Yu *et al.*, 2007
Secale cereale	300 kDa	60 kDa (homopentamer)	Sue *et al.*, 2000
Dalbergia nigrescens	240 kDa	62-63 kDa (heterotetramer)	Chuankhayan *et al.*, 2005
Avena sativa	360 kDa	60 kDa (homohexamer)	Kim *et al.*, 2005
Zea mays	nd	60 kDa (monomer)	Esen, 1992

Prunus avium	70 kDa	68 kDa (monomer)	Gerardi *et al.*, 2001
		Animals	
Achatina fulica	220 kDa	110 kDa (homodimer)	Hu *et al.*, 2007
Homo sapiens	nd	53 kDa	Berrin *et al.*, 2002
Apis mellifera	nd	72 kDa	Pontoh and Low 2002
		Microbes	
Daldinia eschscholzii	nd	64 kDa	Karnchanatat *et al.*, 2007
Aspergillus oryzae	nd	120 kDa	Langston *et al.*, 2006
Aspergillus niger	240 kDa	120 kDa (homodimer)	Watanabe *et al.*, 1992
Stachybotrys Sp.	85 kDa	85 kDa (monomer)	Amouri and Gargouri, 2006
Talaromyces thermophillus	49 kDa	50 kDa (monomer)	Nakkharat and Haltrich, 2006
Scytalidium thermophilum	39 kDa	42 kDa (monomer)	Zanoelo *et al.*, 2004
Chaetomium thermophilum	nd	43 kDa	Venturi *et al.*, 2002

nd, not determined

Proteomic analysis of the polypeptides by MALDI-TOF illustrated their identity as pertaining to fucosyltranferase and β-glucosidase. The former has been discussed in detail at the end of this chapter whilst comparative account of the later is discussed herein.

The molecular mass around 50-60 kDa is the most common for β-glucosidase subunits in plants although the subunit size variation is wider in microbes and animal (40 to 120 kDa) (Table 1). Plant β-glucosidases are more common to occur in multimeric forms- e.g. dimmer (*Strychnos mellodora, Malus domestica*), tetramer (*Dalbergia nigrescens*), pentamer (*Dalbergia cochinchinensis, Secale cereale*), hexamer (*Avena sativa*) etc. The monomeric form of all family 1 β-glucohydrolases is composed of a single-domain $(\beta/\alpha)_8$ barrel with a molecular mass of ~50 kDa (Isorna *et al.*, 2007). Oligomerization is not a strict requirement for stability/activity. However, multimerization appears to improve upon the chances of higher proportions of substrate-enzyme collision resulting into catalytic reaction owing to the presence of more than one active sites on the oligomeric protein.

The *Silybum* petal homodimer β-glucosidase has complimentary molecular mass like *Malus domestica* β-glucosidase (Yu *et al.*, 2007) which has 120 kDa native and 60 kDa M_r for each subunit. Homodimeric β-glucosidases are reported from all three types of organisms including plant *Strychnos mellodora* (200 kDa) (Brandt *et al.*, 2000), animal *Achatina fulica* (220 kDa) (Hu *et al.*, 2007), and fungal enzyme *Aspergillus niger* (240 kDa) (Watanabe *et al.*, 1992).

5.2. pH optima and pH stability

In general, β-glucosidases have acidic pH optima as well as acidic pH stability in plants and to some extent in animals and microbial enzymes too. The pH optima and pH stability results on the selected medicinal plant β-glucosidases joined to emphasize this generality in the hydrolase family enzymes. The *Withania somnifera*

β-glucosidase had optimal activity at pH 4.8 with half maximal activities at pH 4.0 and 7.0 and pH stability of the enzyme was also in the acidic (pH 5.0 to 6.0). Similarly, *Andrographis paniculata* β-glucosidase showed catalytic optimum at pH 5.5 with half maximal activities at pH above 4.5 and 6.5 and pH stability of the enzyme also almost in the same (pH 5.0 to 6.0) range. In the same way, *Silybum marianum* enzyme had optimal activity at pH 5.5 with half maximal activities at pH 4.5 and >6.5 but it retained >20% activity at assay pH 3.5 and 8.0 as a partial deviation from the generality. The enzyme pH stability for the enzyme in pH range 5.0-7.0 matched more with the acidic stability, although it retained about 60% of activity even after 4 h at pH 4.0 and 8.0. It seems acidic micro-milieu is an almost necessity for the appropriate ionization states of the amino acid residues involved in acid-base reaction for the catalysis.

However, some β-glucosidases possess relatively broader pH range of optimal activity that reaches or nears neutrality like *Secale cereale* and *Dalbergia nigrescens* (pH 5.0 to 6.0) (Sue *et al.*, 2000; Chuankhayan *et al.*, 2005) whilst the enzyme from some other plants *Vigna radiata* and *Manihot glaziovii* (Zeng and Elbein, 1998; Yeoh and Wee, 1994) and non-plants like *Homo sapiens, Scytalidium thermophilum* (Berrin *et al.*, 2002; Zanoelo *et al.*, 2004) has been shown to possess pH optima of nearly neutral (6.5). Cassava (*Manihot esculanta*) tuber enzyme was known to have an unusually broad pH range (pH 5.0 to 8.0) of retaining (85%) maximal activity (Keresztessy *et al.*, 2001). More precisely, *Withania* β-glucosidase was noted to be similar to several microbial enzymes, besides few plants like *Citrus sinensis, Dalbergia cochinchinensis* (pH 4.5 and 5.0) (Srisomsap *et al.*, 1996; Barbagallo *et al.*, 2007).

While comparing *Andrographis* and *Silybum* β-glucosidase, similar narrow acidic pH range of optimal activity and stability has been documented for several β-glucosidases of plant and non-plant origin including microbes like *Passiflora foetida,*

Table 2: pH, *p*I and temperature optima of β-glucosidases from different sources.

Source	pH optima	*p*I	Temperature optima (°C)	E_a (kCal.Mol$^{-1)}$	Reference
			Plants		
Withania somnifera	4.8	8.7	40	12.4	Present Work
Andrographis paniculata	5.5	4.0	55	6.80	Present Work
Silybum marianum	5.5	nd	40	8.26	Present Work
Dalbergia cochinchinensis	5.0	nd	nd	nd	Srisomsap *et al.*, 1996
Catharanthus roseus	6.0	nd	50	nd	Luijendijk *et al.*, 1998
Citrus sinensis Osbeck	4.5	nd	60	nd	Barbagallo *et al.*, 2007
Vigna radiate	6.5	nd	37	nd	Zeng and Elbein, 1998
Manihot glaziovii	6-6.5	nd	55	5.7	Yeoh and Wee, 1994
Passiflora foetida	5.5	nd	57	7.3	Yeoh and Wee, 1994
Prunus polystachya	5.5	nd	60	8.2	Yeoh and Wee, 1994
Prunus avium	nd	4.6	nd	nd	Gerardi *et al.*, 2001
Malus domestica	6.0	5.7	70	nd	Yu *et al.*, 2007
Secale cereale	5.0-5.5	nd	25-30	nd	Sue *et al.*, 2000
Dalbergia nigrescens	5.0-6.0	nd	65	nd	Chuankhayan *et al.*, 2005

Zea mays	5.8	5.2	50	nd	Esen, 1992
Animal					
Achatina fulica	5.0	nd	50	nd	Hu *et al.*, 2007
Homo sapiens	6.5	nd	37	nd	Berrin *et al.*, 2002
Apis mellifera	5.0	4.5-4.8	50	nd	Pontoh and Low 2002
Microbes					
Daldinia eschscholzii	5.0	8.55	50	nd	Karnchanatat *et al.*, 2007
Aspergillus oryzae	5.0	nd	60	nd	Langston *et al.*, 2006
Stachybotrys sp.	5.0	nd	50	nd	Amouri and Gargouri, 2006
Scytalidium thermophilum	6.5	6.5	60	nd	Zanoelo *et al.*, 2004
Aspergillus niger	4.5	4.0	50	nd	Watanabe *et al.*, 1992
Chaetomium thermophilum	5.5	7.7	65	nd	Venturi *et al.*, 2002

nd, not determined

and *Prunus polystachya* (5.5) (Yeoh and Wee, 1994), *Secale cereale* (5.0-5.5) (Sue *et al.*, 2000), *Zea mays* (5.8) (Esen, 1992), *Dalbergia cochinchinensis* (5.0) (Srisomsap *et al.*, 1996), *Achatina fulica,* and *Apis mellifera* (5.0) (Pontoh and Low 2002; Hu *et al.*, 2007), *Chaetomium thermophilum* (5.5) (Venturi *et al.*, 2002), *Daldinia eschscholzii, Aspergillus oryzae,* and *Stachybotrys* sp. (5.0) (Amouri and Gargouri, 2006; Langston *et al.*, 2006; Karnchanatat *et al.*, 2007). Whereas, β-glucosidases from some plants like *Vigna radiata* and *Manihot glaziovii* (Yeoh and Wee, 1994; Zeng and Elbein, 1998) and human (Berrin *et al.*, 2002) are known to have a relatively much wider pH range of significant activity with optima nearly neutral (6.5).

5.3. Isoelectric point (*p*I)

The isolelectric point (*p*I) value of 8.7 observed for *Withania somnifera* β-glucosidase is slightly higher than a few reported for plant enzymes but is closer to those reported from some fungi like *Daldinia eschscholzii* and *Chaetomium thermophilum* (8.5 and 7.7) (Venturi *et al.*, 2002; Karnchanatat *et al.*, 2007). Whereas the observed *Andrographis paniculata* β-glucosidase nt (*p*I) value of 4.0 (the acidic *p*I) is mechanistically interesting in terms of ionization of acidic amino acids some of which are considered to participate in catalysis and has been less frequently observed. It has been reported from a few species- e.g. fungus *Aspergillus niger* (4.0) (Watanabe *et al.*, 1992), insect *Apis mellifera* (4.7) (Pontoh and Low 2002), and plant *Prunus avium* (4.6) (Gerardi *et al.*, 2001).

5.3. Thermostability and thermotropic behaviour

The *Withania* and *Silybum* enzymes were catalytically most active at 40 °C and most of the enzymes from diverse sources have moderate to high temperature of optimal activity (50 to 70 °C), whereas *Vigna radiata, Secale cereale* and *Homo sapiens* enzymes have been reported to be most active at lower temperatures (25 to 40 °C)

(Zeng and Elbein, 1998; Sue *et al.*, 2000; Berrin *et al.*, 2002). The enzymes were also observed to be significant stable only upto 40 °C similar to other low thermostability plant glycosidases like papaya and grape (Estibalitz *et al.*, 2001; Lecas *et al.*, 1991). Contrarily, the enzyme from plants like strawberry and *Hevea* enzymes are known to have much improved thermostability (≥60 °C) (Schreier and Schreier, 1986; Selmar *et al.*, 1987). The *Andrographis* enzyme catalyzed reaction rate was maximal at 55 °C suggesting it to belong to the group of β-glucosidases with moderate to high temperature (50 to 70 °C) like those from *Manihot glaziovii, Passiflora foetida, Zea mays, Catharanthus roseus, Achatina fulica, Apis mellifera* (Esen, 1992; Yeoh and Wee, 1994; Luijendijk *et al.*, 1998; Pontoh and Low 2002; Hu *et al.*, 2007) and also including members from microbes- like *Daldinia eschscholzii, Stachybotrys* sp., *Aspergillus niger* (Amouri and Gargouri, 2006; Langston *et al.*, 2006; Karnchanatat *et al.*, 2007). Reports on highly or extremely thermostable β-glucosidases are still restricted to thermophilus microorganisms but matching magnitude surveys of plants from diverse habitats may result in their more thermostable representatives. The mechanism of temperature stability and pH stability are closely related to the structure and amino acid composition of the enzyme. Replacement of amino acid residues of the C-terminal domain is known to significantly affect the heat stability implying that the residues for imparting thermal stability may be distributed over the entire region of the C-terminal domain (Hu *et al.*, 2007).

For all three purified enzymes the Arrhenius plot was single straight line, typical of the enzymes lacking a tight association with membranes. The plot gave estimates of activation energies for *Withania, Andrographis,* and *Silybum* enzymes to be 12.4, 6.80, 8.26 kCal.Mol^{-1}, respectively. Thus, the activation energy of *Withania* was about 1.5 to 2.0 times higher than the two other enzymes and others reported from *Manihot glaziovii, Passiflora foetida, Prunus polystachya* enzymes (Yeoh and Wee, 1994). *Prunus polystachya* leaf and peel β-glucosidase of cassava had an activation

energy of 6.5 kCal.Mol^{-1} while the tuber cortex enzyme gave a value of 5.8 kCal.Mol^{-1} (Yeoh and Wee, 1994).

5.4. Substrate saturation kinetics

For all three plants enzyme the substrate saturation curve for β-*p*NPG was normal hyperbolic suggesting the enzyme to follow typical Michaelis-Menten kinetics of reaction catalysis. Double reciprocal (Line-weaver Burk) plot the K_m values computed for *Withania somnifera, Andrographis paniculata,* and *Silybum marianum* were 0.19, 0.25, and 0.25 mM, respectively. The V_{max} were observed to be 22.85, 9.22, and 130.0 IU for *Withania somnifera, Andrographis paniculata,* and *Silybum marianum* enzyme, respectively. The low K_m value of the enzymes was of the enzyme one of the novel attribute as most of the plant glycosidases reported so far have relatively very high K_m except those amassing prodigal amounts of secondary metabolites (*Narcissus papyraceus, Daucus carota, Citrus sinensis, Manihot* sp.) (Yeoh and Wee, 1994; Konno *et al.*, 1996; Reuveni *et al.*, 1999; Cameron *et al.*, 2001; Keresztessy *et al.*, 2001). Higher affinity of the enzyme imparts significant catalytic functionality even under low substrate availability, typical for the secondary metabolic products. Although, microbes and animal enzymes appear to possess this property more commonly, probably for nutrient and/or befitting ecological (counter) interaction needs (Table 3).

Within the studied plant enzymes, *Silybum* β-glucosidase had highest V_{max} (130.0 IU) which bracketed it with the high V_{max} plant enzymes like *Daucus carota* and *Malus domestica* for which much higher values (2170 and 52.4 IU) has been reported. *Withania* and *Andrographis* enzymes have comparatively very low V_{max} than *Silybum* but relatively higher than that of several reported plant enzymes (Table 3).

Table 3: Substrate saturation kinetics of β-glucosidases from different sources.

Source	K_m, mM	V_{max}, IU	K_{cat}, s^{-1}	K_{cat}/K_m, $M^{-1}.s^{-1}$	Reference
			Plants		
Withania somnifera	0.19	22.85	19.04	100,210	Present Work
Andrographis paniculata	0.25	9.22	9.60	38,400	Present Work
Silybum marianum	0.25	130.0	154.75	619,000	Present Work
Zea mays	0.64	nd	29.5	46,000	Verdoucq *et al.*, 2003
Dalbergia cochinchinensis	5.4	nd	307.0	56,000	Srisomsap *et al.*, 1996
Dalbergia nigrescens	14.7	nd	10.4	707	Chuankhayan *et al.*, 2005
Vanilla planifolia	3.3	11.5	nd	nd	Dignum *et al.*, 2004
Narcissus papyraceus	0.083	25.9	nd	nd	Reuveni *et al.*, 1999
Daucus carota	0.12	2170	nd	nd	Konno *et al.*, 1996
Digitalis lanata	10.5	0.30	nd	nd	May and Kreis, 1997
Prunus polystachya	3.38	nd	nd	nd	Yeoh and Wee, 1994
Prunus dulcis	3.6	nd	nd	nd	Fischer and Peissker, 1998
Manihot glaziovii	0.30	nd	nd	nd	Yeoh and Wee, 1994
Manihot esculenta	0.36	nd	nd	nd	Keresztessy *et al.*, 2001

Citrus sinensis	0.115	0.32	nd	nd	Cameron *et al.*, 2001
Citrus sinensis Osbeck	0.267	2.1×10^{-5}	nd	nd	Barbagallo *et al.*, 2007
Passiflora foetida	3.89	nd	nd	nd	Yeoh and Wee, 1994
Malus domestica	1.2	52.4	54.58	45,483	Yu *et al.*, 2007
Secale cereale	0.90	49.5	51.56	57,288	Sue *et al.*, 2000
Avena sativa	2.4	nd	3090	1,287,500	Kim *et al.*, 2005
			Animals		
Achatina fulica	0.22	203	376	1,709,090	Hu *et al.*, 2007
Homo sapiens	0.38	Nd	3.12	8210	Tribolo *et al.*, 2007
Apis mellifera venticulus	12.0	1.1	1.3	108	Pontoh and Low 2002
Apis mellifera honey sac	11.3	0.76	0.90	79	Pontoh and Low 2002
			Microbes		
Daldinia eschscholzii	1.52	3.21	3.45	2270	Karnchanatat *et al.*, 2007
Cellulomonas biazotea	0.025	4.8	nd	nd	Lau and Wong, 2001
Nectria catalinensis	0.25	0.23	nd	nd	Pardo and Forchiassin, 1999
Aspergillus oryzae	0.29	nd	370	1,275,862	Langston *et al.*, 2006
Aspergillus niger	0.95	39.8	nd	nd	Seidle *et al.*, 2005

Stachybotrys Sp.	0.30	78.0	118	393,333	Amouri and Gargouri, 2006
Scytalidium thermophilum	0.29	13.2	577	1,989,655	Zanoelo *et al.*, 2004
Chaetomium thermophilum	0.76	203	147	193,421	Venturi *et al.*, 2002

nd, not determined

However, kinetic parameters of catalytic constant or turn over number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) provide a means of more realistic comparison of their performances but they have not been commonly computed for several of the enzymes studied so far. *Withania* enzyme had higher catalytic efficiency (100,210 $M^{-1}.s^{-1}$) than other plant enzymes except *Avena sativa* counterpart that has been reported to have a value higher by one order.

While *Andrographis* enzyme had comparatively half K_{cat}/K_m value (38,400 $M^{-1}.s^{-1}$) than *Withania* and about six times less than *Silybum* enzyme, nevertheless, it was relatively higher than several other enzymes from plants (*Dalbergia nigrescens*, 707 $M^{-1}.s^{-1}$), animal (*Homo sapiens*, 8210 $M^{-1}.s^{-1}$; *Apis mellifera*, 108 $M^{-1}.s^{-1}$), and microbes (*Daldinia eschscholzii*, 2270 $M^{-1}.s^{-1}$) (Pontoh and Low, 2002; Chuankhayan *et al.*, 2005; Karnchanatat *et al.*, 2007; Tribolo *et al.*, 2007). Of course, even higher values have been reported some members from these three source types- *Avena sativa*, *Achatina fulica*, *Aspergillus oryzae*, *Stachybotrys* Sp., *Scytalidium thermophilum*, and *Chaetomium thermophilum* (Table 3). The *Silybum* enzyme had much higher catalytic efficiency (619,000 $M^{-1}.s^{-1}$) which is very high than other plant enzymes except *Avena sativa*; microbial enzymes from *Aspergillus oryzae*, and *Scytalidium thermophilum*, and animal sourced β-glucosidase from *Achatina fulica* have 2-4 fold higher K_{cat}/K_m values. However, several microbial and a Jade snail β-glucosidases possess even higher catalytic efficiency (1,709,090 $M^{-1}.s^{-1}$) and *Stachybotrys* Sp. is reported have more than half K_{cat}/K_m (393,333 $M^{-1}.s^{-1}$) (Table 3).

5.5. Substrate specificity

The enzyme substrate specificity of the three enzymes was tested by monitoring the level of catalytic activity displayed by the enzyme towards different artificial substrates with different types of linkages (β or α), different glyco-moieties conjugated and alternate position of the substituent group ($-NO_2$) on the aglycone

moiety. These included β-linked sugars conjugates like *p*-nitrophenyl-β-D-glucopyranoside (β-*p*NPGlu), *p*-nitrophenyl-β-D-galactopyranoside (β-*p*NPGal), *p*-nitrophenyl-β-D-mannopyranoside (β-*p*NPMan), *p*-nitrophenyl-β-D-xylopyranoside (β-*p*NPXyl), *p*-nitrophenyl-β-D-fucopyranoside (β-*p*NPFuc), *p*-nitrophenyl-β-D-lactopyranoside (β-*p*NPLac); α-linked sugars conjugates like *p*-nitrophenyl-α-D-glucopyranoside (α-*p*NPGlu), *p*-nitrophenyl-α-D-galactopyranoside (α-*p*NPGal); aglycone substituent group position variants like *o*-nitrophenyl-β-D-glucopyranoside (β-*o*NPGlu) and *o*-nitrophenyl-β-D-galactopyranoside (β-*o*NPGal).

Interestingly, *Withania* enzyme displayed significant deglycosylating activity only with β-*p*NPGlu and even activity with β-*o*NPGlu was just rudimentary (4%) as compared to that with β-*p*NPGlu. Therefore, the enzyme was almost absolutely substrate specific for only β-linkage and with only glucose as the glycone conjugate. Thus, among plants the novel substrate specificity of *Withania somnifera* β-glucosidase matched with only *Oryza sativa* counterpart (Akiyama *et al.*, 1998). *Andrographis paniculata* enzyme though showed maximal hydrolytic activity towards β-*p*NPGlu but it also had some activity with β-*p*NP-Mannose (16%) and β-*p*NP-Fucose (10%) and negligible activity with β-*p*NP-Xylose and β-*p*NP-Galactose (4-6%) and rudimentary (1.4%) with only α-*p*NP-Glucose among the α-linkage glycosides. Also, little (4%) activity was observed with aglycone nitro-group position variant-position-substituent i.e. *ortho*-NP-Glucose (4%) (Table 4). The enzyme from *Silybum marianum* had best deglycosylating actvity with β-*p*NPGlu but >50% activity was recorded with β-*p*NPFuc. The enzyme had significant activity (28%) with *ortho*-substituted nitrophenyl (aglycone) substrate β-*o*NPGlu and a noticeable 14% activity was observed with change in glycone moiety from glucose to galactose in β-*p*NPGal and only 5% activity with change in sugar moiety configuration from β to α (α-*p*NPGlu). This enzyme has no marked activity with lactose, mannose and xylose conjugates as substrate.

Table 4: Substrate specificity of β-glucosidases from different sources.

Source	*p*NP αGlu	*p*NP βGlu	*o*NP βGlu	*p*NP αGal	*p*NP βGal	*o*NP βGal	*p*NP Man	*p*NP βFuc	*p*NP βXyl	Reference
					Plants					
Withania somnifera	0	100	4.4	0	0	0	00	0	0	Present work
Andrographis paniculata	1.4	100	4.2	-	4.2	0	16.5	9.9	6.1	Present work
Silybum marianum	5.4	100	28.3	0.9	14.1	0.4	0.2	5	0.1	Present work
Manihot glaziovii	nd	100	nd	nd	5	nd	5	108	3	Yeoh and Wee, 1994
Passiflora foetida	nd	100	nd	nd	23	nd	5	233	8	Yeoh and Wee, 1994
Prunus polystachya	nd	100	nd	nd	22	nd	23	158	15	Yeoh and Wee, 1994
Prunus avium	nd	54	100	nd	21	nd	nd	42	1	Gerardi *et al.*, 2001
Oryza sativa	0	100	nd	0	0	nd	0	0	0	Akiyama *et al.*, 1998
Dalbergia nigrescens	nd	100	nd	nd	3.9	nd	0.4	124	7.5	Chuankhayan *et al.*, 2005
Dalbergia cochinchinensis	nd	100	nd	nd	8.9	nd	0.2	124	3.9	Srisomsap *et al.*, 1996
					Animals					
Achatina fulica	0.03	100	nd	1.1	1.6	nd	0.03	0.19	2.9	Hu *et al.*, 2007

Homo sapiens	nd	100	nd	nd	145	nd	nd	88.4	6.1	Berrin *et al.*, 2002
					Microbes					
Daldinia eschscholzii	nd	100	15.5	nd	Nd	nd	nd	nd	7	Karnchanatat *et al.*, 2007
Talaromyces thermophillus	nd	100	nd	nd	nd	nd	nd	nd	nd	Nakkharat and Haltrich, 2006
Scytalidium thermophilum	nd	100	nd	nd	nd	nd	nd	nd	15.7	Zanoelo *et al.*, 2004
Chaetomium thermophilum	nd	100	nd	0	nd	nd	nd	nd	0	Venturi *et al.*, 2002

nd, not determined

Thus, among the enzymes studied *Withania somnifera* leaf enzyme had most stringent substrate restriction whilst that from *Silybum marianum* accepted a wide range of substrates.

Substrate specificity of *Silybum marianum* β-glucosidase matched with *Manihot glaziovii, Passiflora foetida,* and *Prunus polystachya* (Yeoh & Wee, 1994). Overall, the observed relatively stricter substrate specificity of *Withania, Andrographis* and *Silybum* enzymes together with their lower thermostability corroborates the perception that metastable β-glucosidades have more stringent substrate specificity. The observed very significant (>50%) activity with fucose conjugates and noticeable (14%) one with galactose conjugates with *Silybum marianum* was interesting. 'True β-glucosidases' are rare, particularly plant β-glucosidases inherently possess much more β-fucosidase activity than β-glucosidase implying them to be more correctly referred as the former. It is academically more interesting that, several β-glucosidases that lacked significant β-galactosidase but had prodigal β-fucosidase activity, where D-fucose is structurally 6-deoxy-D-galactose with identical patterns of equatorial and axial hydroxyl groups in a pyranose conformation.

Comprehending available structural information the enzymes from a few sources, β-glucosidases may posses fucosidase type activity due to the non-covalent interactions with the C-5 hydroxylmethyl group of the glucosyl residues which are rarely crucial for enzyme activity. The structural/positional differences on acting-sites in glycosides are major cause of enzyme-substrate specificity and β-glucosidase has the highest affinity for a glucoside with a polar group in the *para*-position. Glucosides with a non-polar or no susbtituent in the *para*-position and a methoxyl-group in *ortho*-position are poorly hydrolyzed. Compounds lacking a polar group in the *para*-position and a methoxyl-group in *ortho*-position or that have the glucose attached to the sidechain are not hydrolyzed at all (Dignum *et al.,* 2004). These

inferences have emerged from comparative catalytic and/or structural features of parental enzyme with those of its site-directed mutagenesis generated variants. Similarly, structural characterization of naturally occurring counterparts (like the ones reported here) could add values to these conclusions as it entails evolutionary shaping/ordering (of amino acid residues) for functional catalytic specificity.

Many β-glucosidases/galactosidases have both β-galactosidase and β-glucosidase activities even if they are referred to in articles, books and databases on the basis of dominant/interest activity as either β-glucosidase or β-galactosidase. Galactosidase activity of β-glucosidases is considered to be due to a conserved glutamate residue, down-stream of nucleophilic glutamate, commonly present in all family 1 β-glucosidades to present a bifurcated hydrogen bond to O4 and O6. Conformational freedom of the enzymes to allow the side chain of this glutamate to adapt its position to an axial O4 (besides equatorial O4 in glucose), and thereby enable recognition of galacto-conjugates as well. Probably, higher fucosidase activity, than galactosidase and even glucosidase, known with several β-glucosidases implies that hydrogen bonding with axial O4 alone (fucoside) is catalytically more favorable than O6 and axial O4 (galactoside) or O6 and equatorial O4 (glucoside) conformation in the glycone.

Therefore, contrary to *Andrographis paniculata* enzyme, lack of activities with mannose conjugates as observed with *Withania somnifera* and *Silybum marianum* enzymes implies lack of hydrogen bonding of the relevant amino acid residue side chain with an axial O2 resulting in catalytically unfavorable positioning/docking (or weakened binding) or oxocarbenium transition state formation (Langston *et al.*, 2006). All three enzymes reported herein lacked any significant activity towards mannose conjugates.

Mechanistically, β-glucosidases catalyze anomeric configuration retained hydrolysis of their substrates through nucleophile attacks initiated double–

displacement mechanism with intermediacy of oxocarbenium ion-like transition state involving a acid/base (proton donor) catalytic glutamate residue and a nucleophilic glutamate residue. The two catalytic glutamate residues located at the C-terminal end of β-strands 4 and 7 are housed in the active site placed in a wide cavity along the axis of the eight-fold β/α barrel motif (Isorna *et al.*, 2007). The catalytic glutamate is located close to glycosidic atom and the nucleophile glutamate is located far from the scissile bond and interacts with O3 of glycone. Interaction of the later with O2 reflecs the role of the substrate 2-OH in stabilizing the transition state (oxocarbenium-ion like transition state). A glutamate at about 50 amino acid residue down-stream of nucleophile glutamate presents the bifurcated link to glycosides O4 and O6 (Isorna *et al.*, 2007).

The structure-catalytic activity rational for the strict glycone specificity is little known as most of the structurally characterized β-glucosidases so far show relaxed substrate specificity for glycone. Thus, it may be insighting to hypothesize that the members like *Withania* and *Oryza* β-glucosidases that lack galactosidase and fucosidase activity may have a catalytically unsuitable substitution at this glutamate residue and could be good candidates to advance molecular understanding of structure-catalysis aspects of the enzyme. Similarly, lack of activities with mannose implies lack of hydrogen bonding of the relevant amino acid residue side chain with an axial O2 resulting in catalytically unfavorable positioning/docking (or weakened binding) or oxocarbenium transition state formation (Langston *et al.*, 2006). It has been shown that mutant enzymes with substitution of a conserved tryptophan (Trp^{345}) into methionine and alanine had, respectively, 100 and 2000 times reduced β-galactosidase activity. Amine group of this tryptophan indole ring is located near the O6 of the sugar and can act as a stacking platform in the aglycone pocket, key to ensure glucosidic bond positioning in an orientation favourable for attack by the two catalytic residues glutamates. Aglycone moiety is sandwitched between selected

tryptophan and phenylalanine residues suggesting that conformation of these hydrophobic amino acids and the shape formed within these determine aglycone recognition and substrate specificity. Thus, severe hindrance (95.6%) of catalytic pathway observed with *o*NPGlu probably indicates that the presence of bulky and electrostatic groups like $-NO_2$ near (*ortho*) *vicinial* to the site of deglycosylating site (-*O*-glycone) might be associated with interfered hydrophobic interactions at the catalytic site (Isorna *et al.*, 2007; Hu *et al.*, 2007).

5.6. Inhibition kinetics

The *Withania somnifera* enzyme was strongly inhibited by D(+)-glucono-1,5-lactone with more than 50% inhibition at 100 µM and more than 90% inhibition at 1.0 mM. Similarly, rice β-glucosidase has been reported to be inhibited by 97% at 1.0 mM D-gluconic acid lactone concentration (Akiyama *et al.*, 1998). In a more or less similar fashion, *Silybum marianum* β-glucosidase inhibition studies with D-glucono-1,5-lactone showed that the enzyme was mildly sensitive to inhibition with more than 50% inhibition at 5 mM and almost complete inhibition was observed above 50 mM concentration. The inhibition pattern shows that at initial gluconolactone (1-5 mM) concentration the activity is steeply inhibited thereafter a saturation kind of situation is observed and hence in the range of 30-60 mM gluconolactone concentration only 12% loss in activity (87-99%) was recorded.

Among all three studied enzymes, *Andrographis panicuata* β-glucosidase was *almost* insensitive to its inhibitory action. It could inhibit about 50% at very high millimolar (10 mM) concentration. Complete loss of activity was noted at 50 mM D-gluconolactone concentration. These inhibitor concentrations are too excessive to foresee their physiological relevance or to qualify for significant protein-ligand interactions. Commonly β-glucosidases are almost inhibited by D-glucono lactone in micromolar concentrations (Akiyama *et al.*, 1998). The examples of this property

enzymes are so far restricted to two secondary metabolic pathway specific β-glucosidases from two indole alkaloid producer plants of Apocyanaceae - strictosidine glucosidase of *Tabernaemontana divericata* (Luijendijk *et al.*, 1998) and raucaffricine-β-D-glucosidase (SG) from *Rauwolfia serpentine* (Schubel *et al.*, 1986) Whereas, the SGs from another two other producer of the alkaloids (*Catharanthus roseus*, Apocyanaceae; *Strychnos mellodora*, Loganiaceae) have been shown to be strongly inhibited by gluconolactone (Hemscheidt and Zenk, 1980; Brandt *et al.*, 2000). Recently, a β-glucosidase from thermophillic fungus (*Talaromyces thermophillus*) has been shown to be similarly insensitive to D(+)-glucono lactone inhibition (Nakkharat and Haltrich, 2006). The enzymes with intermediary level of sensitivity to the inhibition have also been noticed with the enzyme from a few plants- *Manihot glaziovii, Passiflora foetida* and *Prunus polystachya* (Yeoh and Wee, 1994). Thus, inhibitory action by D(+)-gluconic acid δ-lactone, considered due to structural similarity to an intermediary compound in the catalytic reaction (Murray *et al.*, 2004), may be more specific than perceived.

Analysis of the inhibition kinetics proved that D(+)-gluconic acid δ-lactone acted as an uncompetitive inhibitor and the inhibition constant (K_i) were estimated to be 0.21, 5.6, and 5.0 mM for *Withania, Andrographis,* and *Silybum* β-glucosidase. Inhibition constants were derived from the plot drawn between inhibition constant and inverse of activity at different inhibitor concentrations ($1 / V_i$). K_i, the inhibitor concentration of half of maximal activity for the enzymes were varied considerably within the enzymes examined- millimolar for *Andrographis* and *Silybum* enzymes and micromolar for *Withania* counterpart. Variation in K_i values has important implications in analyzing and understanding inhibitor-enzyme *versus* enzyme-substrate interactions in the perspectives of variations in the relevant amino acid residues.

5.7. Effect of Glucose

Glucose and some other monosaccharides have been reported to have moderate inhibitory effect in some β-glucosidases from plant, animal and microbial origin. Maize β-glucosidase (Babcock and Esen, 1993) was poorly inhibited by glucose like almond and cassava enzymes (Yeoh and Wee, 1994).

Contrarily, glucose as such was found to have no effect on *Withania* enzyme activity at low concentrations (0.1 to 2.0 mM) and reduced activity by mere 10% per 10 mM increase in glucose concentration beyond 4.0 mM. However, *Andrographis* enzyme showed negligible (8-10%) inhibition at concentrations up to 0.5 mM and the inhibition remained very low (16-20%) even when glucose concentration in the assay mixture was enhanced to several fold higher level (upto 20 mM). Further, even at 40 mM glucose, the inhibition was merely 23%. Thus, the *Andrographis paniculata* leaf β-glucosidase does not share glucose inhibition property with the other counterparts. In more stringent way, *Silybum* enzyme activity was not affected by 2 to 40 mM experimental glucose concentrations.

The fungal strain *Talaromyces thermophillus* showed more then 40% inhibition in β-glucosidase activity at 10 mM glucose concentration (Nakkharat and Haltrich, 2006); similarly D-glucose was reported to be significant competitive inhibitor of *Aspergillus oryzae* family 3 β-glucosidase (Langston *et al.*, 2006). *Aspergillus niger* β-glucosidase was also reported to be inhibited by glucose but the enzyme from different yeast strains was found to be activated upto 10-15% at low concentration (5-15%) and then inhibited at concentrations over 15% glucose (Barbagallo *et al.*, 2004). Another fungal β-glucosidase from *Scytalidium thermophillum* was activated to 2-fold at 50 mM glucose concentration (Zanoelo *et al.*, 2004).

5.8. Effect of cations and chelating agents

Withania somnifera and *Silybum marianum* β-glucosidase activities were unaffected by several divalent (Mn^{2+}, Ca^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and Co^{2+}) and monovalent (Li^{+}, K^{+} and Na^{+}) cations at low concentrations (1 mM) but their higher concentrations (5 mM) were slightly inhibitory. But Mg^{2+} and Fe^{2+} showed strong inhibition of *Withania* enzyme activity. Similarly, *Andrographis paniculata* enzyme activity was unaffected by K^{+}, Na^{+} (monovalent), Zn^{2+}, Mg^{2+}, and Cu^{2+} (divalent) cations at 1 mM concentration and negligibly at 10 mM. But Li^{+} showed moderate inhibition of 10% and 25% at 1.0 mM and 10 mM concentrations, respectively. Among divalent cations, Mn^{2+} and Ca^{2+} were moderately inhibitory for *Andrographis* β-glucosidase even at high concentration (10 mM). The *Silybum marianum* β-glucosidase was not markedly affected by monovalent cations like Li^{+}, K^{+}, and Na^{+} at either of three concentrations (1.0, 2.5 and 5.0 mM) while Ag^{+} was proven to be highly inhibitory in nature. Similarly, divalent cations have more or less no effect on *Silybum* enzyme activity except Co^{2+}, Mn^{2+}, Cu^{2+}, and Cd^{2+} which inhibited 30-40% activity at 5 mM concentration while their 1.0 and 2.5 mM concentrations showed almost none effect on enzyme activity.

On *Withania* β-glucosidase Fe^{2+} was strongly inhibitory but an activator for the *Andrographis* and *Silybum* enzymes. More imminently, Fe^{3+} activated the *Withania* and *Silybum* enzymes but inhibited *Andrographis* β-glucosidase. Ag^{+}, the well know inhibitory metal ion for β-glucosidases strongly inhibited *Withania, Andrographis* and *Silybum* enzymes but by differential extent- e.g. 42%, 75% and 94%, respectively, at 1 mM. Another strong cationic inhibitor for enzyme was Hg^{2+} that inhibited *Withania* enzyme completely but by 43% and 92%, respectively, *Andrographis* and *Silybum* β-glucosidases at 1 mM concentration. More or less similar results have been reported for enzyme from wood decaying fungus *Daldinia eschscholzii* (Karnchanatat *et al.*, 2007) but its counterpart from a strain of fungus *Talaromyces thermophillus*

(Nakkharat and Haltrich, 2006) has been reported to be activated by the divalent cations Mg^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+} and Co^{2+} at their concentrations upto 10 mM but inhibited by Cu^{2+} and Ag^{+}. The inhibition properties of the *Withania somnifera, Andrographis paniculata* and *Silybum marianum* enzyme were shared by *Secale cereale* (Sue *et al.*, 2000) and almond/apple (Yu *et al.*, 2007) counterparts, respectively.

Chelating agents like EDTA and EGTA did not influence β-glucosidase activity in *Withania somnifera, Andrographis paniculata* and *Silybum marianum* by any significant extent as there is no cationic involvement in the catalytic activity of enzyme.

5.9. Effect of thiol directed reagents

Thiol directed reagents like iodoacetamide, iodoacetate, benzidine and *p*-chloromercuribenzoate did not show any significant inhibition of the *Withania somnifera, Andrographis* and *Silybum* β-glucosidases activity suggesting lack of –SH groups in the catalysis. This observation confirms to the bonding interactions of ligands at the active site of some of the few β-glucosidases of plant and human origin crystallized and characterized in structural details of catalysis as cysteine residues form neither the active site nor are involved in other critical bonding/folding for substrate positioning/orientation (Isorna *et al.*, 2007).

5.10. Effect of organic solvents

The effect of miscible organic solvents (ethanol, methanol, acetone and dimethyl sulphoxide) on β-glucosidase activity was observed at their 10 to 50% (v/v) concentration. Dimethyl sulphoxide (DMSO) was found to reduce the hydrolytic activity of *Withania somnifera* β-glucosidase by 30 and 90% at 10 and 50% DMSO concentrations. The *Andrographis paniculata* β-glucosidase hydrolytic activity was reduced by 37 and ≥90% at 10 and 50% concentrations of DMSO. While DMSO has

very different behavior on *Silybum marianum* enzyme activity and showed no effect up to 20% concentration and a sudden loss of about 60% activity was recorded at 30% concentration whereas 40% concentration caused almost complete inhibition of enzyme activity. Almost identical pattern was observed with other solvents except that inhibition values with acetone were higher for *Withania* β-glucosidase while *Andrographis* enzyme was found to be mostly affected (inhibited) by ethanol and methanol was least effective. Whereas, methanol was found to have highly diminishing effect on *Silybum* β-glucosidase activity which inhibited about 40% activity at 5 % and more than 92% activity at 30% concentration. Ethanol and acetone also have similar activity decreasing fashion of but with a low tendency.

Organic solvent (low polarity) environment mediated diminution of β-glucosidases is understandable as the hydrolytic activity of the enzyme requires considerable aqueous micromilieu for nucelophile attack and acid/base catalysis of hydrolysis at the glucosidic bond. The non-aqueous environment (organic solvent) is conducive to gain reverse (transglycosylating) activity for the enzyme.

5.10. Transglycosylation

A priori to assess the transglycosylating catalytic activity of the selected medicinal plants β-glucosidase, lower aqueous micromilieu was generated through DMSO (10%). The transglycosylation reaction was carried on different aglycone acceptors like methanol, ethanol, propanol, butanol, decanol, geraniol, linalool, menthol, withaferin A, quercetin, and ursolic acid and cellobiose or *p*NPG were used as the glycosyl donor. Thin layer chromatographic analyses of the catalytic reaction products were monitored and compared with appropriate controls. In case of *Withania, Andrographis* and *Silybum*, glycoside formation was observed (R_f 0.27) only with geraniol as the aglycone acceptor and *p*NPG as the glycosyl donor. In addition, *Silybum* β-glucosidase showed transglucosylation reaction also with

withaferin-A and the product formed was obtained as withaferin-A glucoside (R_f 0.14).

Almond β-glucosidase has been reported to use both cellobiose and gentiobiose as glucosyl donors while Thai rosewood β-glucosidase has been demonstrated to use only gentiobiose and cassava β-glucosidase could not use either (Svasti *et al.*, 2003). Although, going by the number of plant β-glucosidase characterized from plants, tranglycosylating property has been studied with a bare few, contrary to microbial situation. Therefore, plant counterparts need attention as (i) β-glucosidases and glucosyl transferases are considered to be evolved from a single ancestral gene and their catalytic site involves analogous hydrophobic interactions, (ii) glycosyltransferase catalyzed glycosylation the reaction is energy driven and involves abundant supply of expensive sugar donor. Trasnglycosylation activity of β-glucosidases may also be functionally relevant *in vivo*, at least under certain extrinsic or intrinsic situation, as it is energy conserving alternative. Moreover, protein and metabolic engineering crafted high catalytic turnover transglycosylating β-glucosidase could be a good proxy enzyme as glucosyltransferases (GTs) of plant secondary metabolism as GTs *per se* have very poor abundance in the tissues (Svasti *et al.*, 2003).

5.11. Proteomic analysis of *Silybum marianum* petal β-glucosidase

The β-glucosidase preparation from *Silybum marianum* petals at advanced stage of purification contained two polypeptodes: 74.1 kDa and 67.5 kDa. Further characterization including cognizance of the MW of the polypeptide with that of the native enzyme suggested that only smaller polypeptide (67.5 kDa) was related to β-glucosidase whilst 74.1 kDa polypeptide appeared to be a co-purified contaminant. Characterization of the 67.5 kDa polypeptide by MALDI-TOF-TOF revealed it to be a putative β-glucosidase. The peptide fragment amino acid sequence analysis and its

multiple sequence analysis through BLAST and ClustalX revealed it to contain at least two sequence motifs VTPSNEVH and KRSEESNF that very strongly matched with β-glucosidases from databases. The multiple sequence alignment revealed that these two sequence motifs shared highly conserved match with *Stigmatella aurantiaca* 1,4-β-D-glucan glucohydrolase D (VTPNNEVT) (Nierman, 2006), *Bacillus thuringiensis* 6-aminohexanoate-dimer hydrolase (ATPSNEIH) (Han *et al.*, 2006), *Nitrosococcus oceani* ATCC 19707 Glycoside hydrolase, family 57 (NTSEESNF) (Klotz *et al.*, 2006), *Bacillus halodurans* β-glucosidase (KRSMESGV) (Takami *et al.*, 2000). Among them an amino acid T^{767}, P^{768}, N^{771}, and E^{772} from *Stigmatella aurantiaca* and T^{396}, P^{397}, N^{398}, and E^{399} from *Bacillus thuringiensis* was highly conserved (matching completely) in first sequence motif of *Silybum marianum* β-glucosidase. Similarly, S^{246}, E^{248}, and S^{249} from *Nitrosococcus oceani* and S^{386}, E^{388}, and S^{389} from *Bacillus halodurans* showed complete match with that of second sequence motif of *Silybum marianum* β-glucosidase.

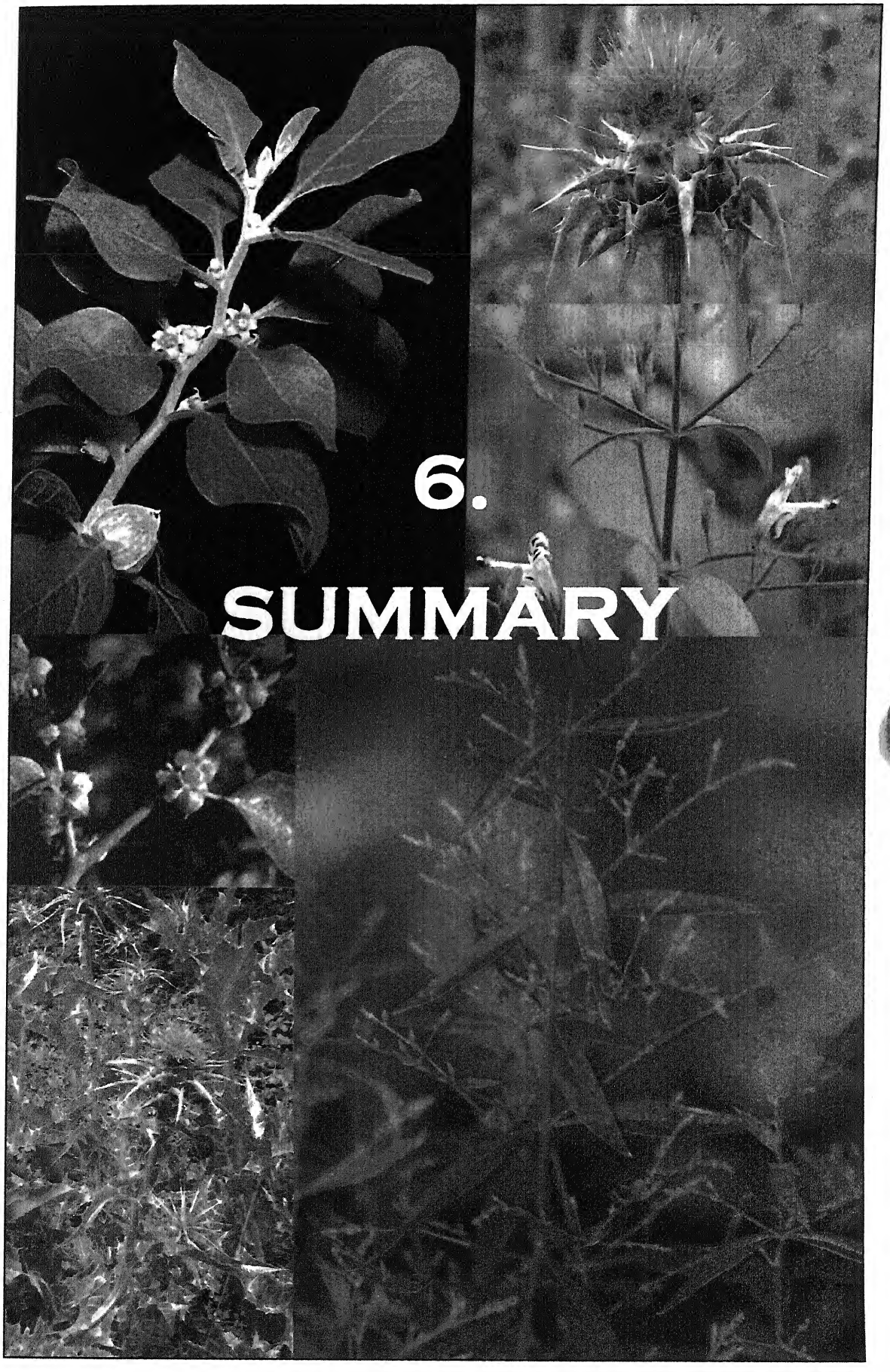
5.12. Proteomic identification of a fucosyltransferase from *Silybum marianum* petals

Fucosyltransferase catalyzes transfer of L-fucose from a GDP-fucose (Guanosine diphosphate-fucose) donor to an acceptor leading to biosynthesis of fucosides. The acceptor substrate can be another sugar such as a core GlcNAc (*N*-acetylglucosamine) sugar as in the case of *N*-linked glycosylation, or a protein, as in the case of *O*-linked glycosylation produced by *O*-fucosyltransferase. There are various fucosyltransferases in mammals, the vast majority of which are located in the golgi apparatus however *O*-fucosyltransferases have recently been shown to be localized to ER also. Compared to animals, very few fucosyltransferases are known and far little characterized from plant world.

The 74.1 kDa polypeptide which appeared to be a co-purified protein characterized by MALDI-TOF revealed it to be a putative fucosyltransferase. The peptide fragment analysis revealed to contain at least two sequence motifs (YYEAYLSHADEK and TTPDPSCGR). These two motifs were shared highly conservatively with fucosyltransferases of *Arabidopsis thaliana* (xyloglucan fucosyltransferase and putative fucosyltransferase 10) (Perrin *et al.*, 1999; Sarria *et al.*, 2001), *Oryza sativa* (putative xyloglucan fucosyltransferase and galactoside 2-α-L-fucosyltransferase) (Sasaki *et al.*, 2002; Yu *et al.*, 2005), *Populus tremula* x *Populus alba* (α-1,2-fucosyltransferase) (Costa *et al.*, 2007). It also showed less but significant similarity even with non-plant fucosyltransferases like *Homo sapiens* (galactoside 3(4)-L-fucosyltransferase, α-(1,3)-fucosyltransferase, galactoside 2-α-L-fucosyltransferase 2) (Kukowska-Latallo *et al.*, 1990; Natsuka *et al.*, 1994; Kelly *et al.*, 1995). In view of very few reports and little functional characterization of plant fucosyltransferases, the identification of the conserved domains of plant fucosyltransferases can be highly useful for cloning their gene in heterologous systems and catalytic and functional characterization. It could also be useful to identify their counterparts involved in fucosylation of small molecules (e.g. secondary metabolites) little known so far.

In summation, the catalytically diverse *Withania somnifera, Andrographis paniculata* and *Silybum marianum* β-glucosidase including some novel features add to the impetus of the exploring tremendous potential of the plant β-glucosidase to develop a library of catalytically characteristic members of the family out of their huge repertoire expected to exist in plants for not only structure functional understanding but also for biotransformation applications. Considering that enzymes with novel functions and properties can be obtained either by searching the largely unknown natural species or by improving upon the currently known

ones by site directed mutagenesis at the sites of potential interest/catalytic function. Though, the later approach is more suitable and mainly followed, currently, for creating properties for which natural evolutionary processes are unlikely to have selected, the later is important for understanding functional selectivity/flexibility in real life. Specifically, *Andrographis paniculata* β-glucosidase is a novel member of the family in terms of its substrate saturation kinetics, gluconolactone and glucose inhibition and explorable tranglycosylating activity. These attributes qualify it to be added to the knowledge library of β-glucosidase for academic understanding and potential biotransformation applications. The high expression of β-glucosidase in petals of *Silybum marianum* showed that it has characteristic novelties which could be of a key enzyme in floral development and can be explored for other metabolic functions within plant secondary metabolism.



6.

SUMMARY

β-D-Glucoside glucohydrolases (3.2.1.21) or β-glucosidases constitute a group of well characterized, biologically important enzymes that catalyze the transfer of glycosyl group between oxygen nucleophiles. Under physiological conditions, such a transfer reaction generally results in hydrolysis of a β-glucosidic bond linking carbohydrate residues in aryl-, amino-, or alkyl-β-D-glucosides, cyanogenic glucosides, short chain oligosaccharides and disaccharides. Under some defined conditions, the reverse of hydrolysis, that is, synthesis of glycosyl-bond between different molecules can occur. This takes place *via* two different modes, reverse hydrolysis and transglycosylation.

β-Glucosidase occurs ubiquitously in living organisms from bacteria to highly evolved plants and animals, and performs diverse functions in them. In bacteria and fungi, β-glucosidases mainly constitute a part of the cellulase enzyme system and are responsible for the hydrolysis of short chain oligosaccharides and cellobiose (resulting from the synergistic action of endoglucanases and cellobiohydrolases) into glucose in a rate-limiting step. In insects and plants, they are implicated in the release of cyanides from cyano-glucoside precursors which is a part of a defense mechanism displayed in these systems. Furthermore, its functions in plants are much more diverse including the hydrolysis of phytohormone precursors, pigment metabolism, seed development, and biomass conversion. Several β-glucosidases of plant origin are highly substrate specific, such as those having selective specificity towards glyco-conjugates of sapogenins, hydrojuglone, cinnamyl alcohols, cyanogenic metabolites, flavones and isoflavones. In addition, plants contain many β-glucosidase whose functions are not fully understood including glucosides of vitamins, naphthquinone derivatives and many others. In humans, membrane-bound lysosomal acid β-glucosidase is implicated in Gaucher's disease as the cells deficient in this enzyme are unable to hydrolyze glycosylceramides. To an advanced approach of therapeutics, Gaucher patients are administered intravenous injection of purified β-glucosidase obtained from human placenta.

As these enzymes occur in all kingdoms of life, they can also serve as useful markers for studying evolution of enzyme specificity and carbohydrate metabolism.

At present there is no well-defined method for the classification of these enzymes. However, on the basis of substrate specificity, β-glucosidases have been grouped into three classes, namely, (i) aryl-β-glucosidases, (ii) true cellobiases, and (iii) broad substrate specificity enzymes, which are active on a wide variety of substrates. In another classification scheme based on sequence and folding similarities the classification of β-glucosidases along with other carbohydrases have been assigned to various families under the glycosylhydrolase category and out of 88 such families defined, β-glucosidases have been placed in family 1 and family 3. Both these families comprise retaining enzymes that hydrolyze their substrates with net retention of anomeric configuration that occurs *via* a double-displacement mechanism. Family 1 of glycosylhydrolases includes β-glucosidases from archaebacteria, plants, and mammals. The crystal structures have been solved for a few family 1 β-glucosidases, which are also designated as members of the 4/7 super family with a common eightfold β/α barrel motif, consisting of similar amino acid sequences at the active site. Family 3 comprises β-glucosidases of some bacterial, mold, and yeast origin.

The applications of β-glucosidases require a large-scale production of the enzymes and a detailed knowledge of functionality and catalytic and regulatory versatility. In this regard, exploration of huge repertoire of β-glucosidases existent in plants particularly medicinal and aromatic plants is required as they have potential role in metabolic management of secondary metabolites/xenobiotics in coordination/complementation of glucosyltransferases. Their catalytic and kinetic variability may have strong bearing with the structural features and functionalities. In medicinal and aromatic plants, their metabolic recruitment in management of secondary metabolites is implicated as the processes of glucosylation and deglucosylation

also involves a substantial change in the polarity of the compounds- glycosides being much more polar (water soluble) than aglycones. Therefore, a comparative knowledge of the catalytic and molecular properties of the enzyme from these plants and other bioresources is of highly academic significance, besides potential applications.

In *Withania somnifera* as well, some of withanolides are encountered in glucosylated form (sitoindosides) thereby involving a role for relevant metabolic steps. Similarly some of the diterpenoids of *Andrographis paniculata* are also glucosylated and the relative proportion of the glucosylated and deglucosylated andrographolide is ontogeny dependent. Thus, it is important to know the role of β-glucosidase / glucosyl transferase in the process. Silymarin, the main oil component in the seeds of *Silybum marianum* was found to be a composition of a large number of flavonolignans including silybin, isosilybin, silydianin and silychristin. Silybin glycosides are established glycoside from *Silybum* which exhibit stronger antioxidant activity. The enzyme β-glucosidase is present in high abundance in petals of the flower which offers to concentrate on the glycosides and glucosidase study of the plant.

Therefore this study, explored the comparative physico-kinetic characteristics of purified β-glucosidases from the selected medicinal plants. Key catalytic and kinetic characteristics and physical features of the purified enzymes from the selected medicinal plants have been comparatively comprehended with their counterparts from other plants, microbes and animals.

The purified enzymes exposed vast physico-kinetic diversity which is insighting for understanding the catalytic mechanism of β-glucosides. The major experimental observations and inferences on catalytic features of the purified enzyme revealed by the study are briefed as under;

Withania somnifera (Solanaceae) leaf β-glucosidase has been purified to homogeniety through a sequence of ammonium sulfate precipitation, gel filtration and ion-exchange column chromatography and characterized for its

physical and kinetic properties. The key catalytic properties of the enzyme included an acidic pH optima (4.8), alkaline *p*I (8.7), meso-thermostabity, small size, monomeric structure with native and subunit molecular weight of about 50 kDa, high affinity for substrate (K_m) for pNPG (0.19 mM), high (100,210 $M^{-1}.s^{-1}$) catalytic efficiency (K_{cat}/K_m). The mesostable enzyme had a stringent substrate specificity restricted to only β-linked gluco-conjugate. The enzyme is optimal active at 40 °C with 12.4 $kCal.Mol^{-1}$ activation energy and was highly sensitive to D-gluconic acid lactone inhibition (94% at 1 mM) with an apparent K_i 0.21 mM. The enzyme could catalyze transglucosylation of geraniol with *p*NPG as glucosyl donor but not with cellobiose.

A gluconolactone inhibition insensitive β-glucosidase from *Andrographis paniculata* (Acanthaceae) leaf was isolated, homogeneity purified and characterized. The 60 kDa enzyme was monomeric and had acidic pH optima (5.5) and *p*I (4.0), had meso-thermostabity and high temperature (55 °C) of optimal catalytic activity with E_a of 6.8 $kCal.Mol^{-1}$. Substrate saturation kinetics of the enzyme for pNPG revealed its K_m and catalytic efficiency (K_{cat}/K_m) estimates of 0.25 mM and 38,400 $M^{-1}.s^{-1}$, respectively. Substrate specificity of the enzyme was restricted to β-linked gluco-, manno- and fuco-conjugates. Insensitivity to inhibition by gluconolactone was evident from very little inhibition at millimolar concentrations. The enzyme showed transglucosylating activity with geraniol as acceptor and *p*NPG as glucosyl donor.

A high abundant β-glucosidase from petals of *Silybum marianum* (Asteraceae) has been purified and characterized for its physico-kinetic properties. The native enzyme was of 135 kDa molecular weight with homodimeric structure of 67.5 kDa two subunits. The biochemical properties of the enzyme comprehend to acidic pH optima (5.5), meso-thermostabity, with β-linked substrate specificity restricted to only β-linked gluco-conjugate and a marked activity with D-fucose is observed. The enzyme has high affinity for *p*NPG with K_m 0.25 mM, V_{max} 130.0 IU and a very high catalytic efficiency

(K_{cat}/K_m) 619,000 $M^{-1}.s^{-1}$ was estimated. The thermal catalytic optimal of enzyme is 40 °C with an activation energy 8.26 kCal.Mol^{-1}. The enzyme has medium sensitive to D-gluconic acid lactone inhibition (57% at 5 mM) with an apparent K_i 5.0 mM and no effect of glucose on enzyme activity could be detected. The transglucosylation activity of enzyme has exceptionally worked for transforming geraniol and withaferin-A aglycone molecules to their corresponding glucosides using *p*NPG as glucosyl donor but cellobiose could not be used as glycosyl donor.

Corroborating from the observations, the subunit and native molecular weight β-glucosidases purified from leaf of *Withania somnifera* and *Andrographis paniculata* were 50 and 60 kDa, respectively whereas native molecular weight of the enzyme from *Silybum marianum* petal was twice larger (135 kDa) with 67.5 kDa two subunits. Coincidently, the final purified enzyme preparation from *Silybum* was showed two bands on SDS-PAGE gel, - the larger (74.1 kDa) and smaller (67.5 kDa) that grossly constituted 60% and 40% of the protein in the preparation, respectively. Proteomic analysis of the polypeptides by MALDI-TOF elucidated their identity as pertaining to fucosyltranferase (74.1 kDa) and β-glucosidase (67.5 kDa).

The pH optima and pH stability profile of the selected medicinal plant (*Withania somnifera, Andrographis paniculata* and *Silybum marianum*) β-glucosidases joined to emphasize this generality in the hydrolase family enzymes. The result obtained showed that it seems acidic micro-milieu is an almost necessity for the appropriate ionization states of the amino acid residues involved in acid-base reaction for the catalysis. The *Withania* and *Silybum* β-glucosidase were catalytically most active at 40 °C and most of the enzymes from diverse sources were reported to have moderate to high temperature of optimal activity (50 to 70 °C), nevertheless, the *Andrographis* enzyme catalyzed reaction rate was maximal at 55 °C which advocated the wide thermal catalytic optima of β-glucosidases. However, highly or extremely thermostable β-glucosidases are still restricted to

thermophilus microorganisms but matching magnitude surveys of plants from diverse habitats may result in their more thermostable representatives.

Among studied plant enzymes, *Silybum* β-glucosidase had highest catalytic efficiency followed by *Withania* and *Andrographis* enzymes. *Withania* and *Andrographis* enzymes have comparatively very low V_{max} than *Silybum* but relatively higher than that of several reported plant enzymes. Higher affinity of the enzyme imparts significant catalytic functionality even under low substrate availability, typical for the secondary metabolic products. Although, microbes and animal enzymes appear to possess this property more commonly, probably for nutrient and/or befitting ecological (counter) interaction needs. The *Withania* enzyme had most stringent substrate restriction whilst that from *Silybum* accepted a wide range of substrates keeping *Andrographis* enzyme in midst of substrate specificity.

The enzyme inhibition studied using D(+)-glucono-1,5-lactone showed very interesting pattern; *Withania somnifera* enzyme was strongly inhibited by micromolar concentration and in a more or less similar fashion, *Silybum marianum* β-glucosidase was mildly sensitive to inhibition. Whereas, *Andrographis panicuata* β-glucosidase was *almost* insensitive to its inhibitory action. Commonly β-glucosidases are reported to be almost inhibited by D(+)-glucono lactone in micromolar concentrations. Analysis of the inhibition kinetics revealed the inhibition constant (K_i) for enzymes which was least for *Withania* followed by *Silybum* and *Andrographis* β-glucosidase, in increasing fashion. Variation in K_i values has important implications in analyzing and understanding inhibitor-enzyme *versus* enzyme-substrate interactions in the perspectives of variations in the relevant amino acid residues.

Accessing the effectors for enzyme activity; glucose (monosaccharide), metal ions, chelating agents, and thiol directed reagents were screened. The result obtained exposed that glucose as such was found to have no effect on *Withania* enzyme activity at low concentrations and slightly reduced activity at higher

glucose concentration. The *Andrographis* enzyme showed negligible/mild inhibition at increasing glucose concentrations. Whereas, *Silybum* β-glucosidase showed no effect of glucose at any experimental concentration. However, glucose and some other monosaccharides have been reported to have moderate inhibitory effect in some β-glucosidases from plant, animal and microbial origin. *Withania somnifera* and *Silybum marianum* β-glucosidase activities were unaffected by several divalent and monovalent cations at low concentrations (1 mM) but their higher concentrations (5 mM) were slightly inhibitory. *Andrographis paniculata* enzyme activity was unaffected by K^+, Na^+ (monovalent), Zn^{2+}, Mg^{2+}, and Cu^{2+} (divalent) cations at 1 mM concentration and negligibly at 10 mM. The *Silybum marianum* β-glucosidase was markedly unaffected by monovalent cations like Li^+, K^+, and Na^+ at either experimental concentrations. Fe^{2+} was found to be strong inhibitor for *Withania* β-glucosidase but an activator for the *Andrographis* and *Silybum* enzymes. More imminently, Fe^{3+} activated the *Withania* and *Silybum* enzymes but inhibited *Andrographis* β-glucosidase. The well known β-glucosidase inhibitor metal ion Ag^+, also inhibited *Withania, Andrographis* and *Silybum* enzymes but by differential extent. Hg^{2+} was found to be another strong cationic inhibitor for enzyme which inhibited *Withania, Andrographis* and *Silybum* β-glucosidases at low concentration. Chelating agents like EDTA and EGTA did not influence β-glucosidase activity in *Withania, Andrographis* and *Silybum* by any significant extent as there is no cationic involvement in the catalytic activity of enzyme. Similarly, thiol directed reagents like iodoacetamide, iodoacetate, benzidine and *p*-chloromercuribenzoate did not show any significant effect on the *Withania, Andrographis* and *Silybum* β-glucosidases activity suggesting lack of –SH groups in the catalysis.

The transglycosylation reaction was successfully catalyzed by all three plant enzymes, wherein, *Withania, Andrographis* and *Silybum* enzymes showed the formation of glycoside only with geraniol as the aglycone acceptor whereas *Silybum* β-glucosidase could also use withaferin-A as an aglycone acceptor. The

glycosyl donor used for transglycosylation was *p*NPG for enzymes but in none of the cases cellobiose could mediate transglycosylation as the glycosyl donor. Trasnglycosylation activity of β-glucosidases may also be functionally relevant *in vivo*, at least under certain extrinsic or intrinsic situation, as it is energy conserving alternative. Moreover, protein and metabolic engineering crafted high catalytic turnover transglycosylating β-glucosidase could be a good proxy enzyme as glucosyltransferases (GTs) of plant secondary metabolism as GTs *per se* have very poor abundance in the tissues.

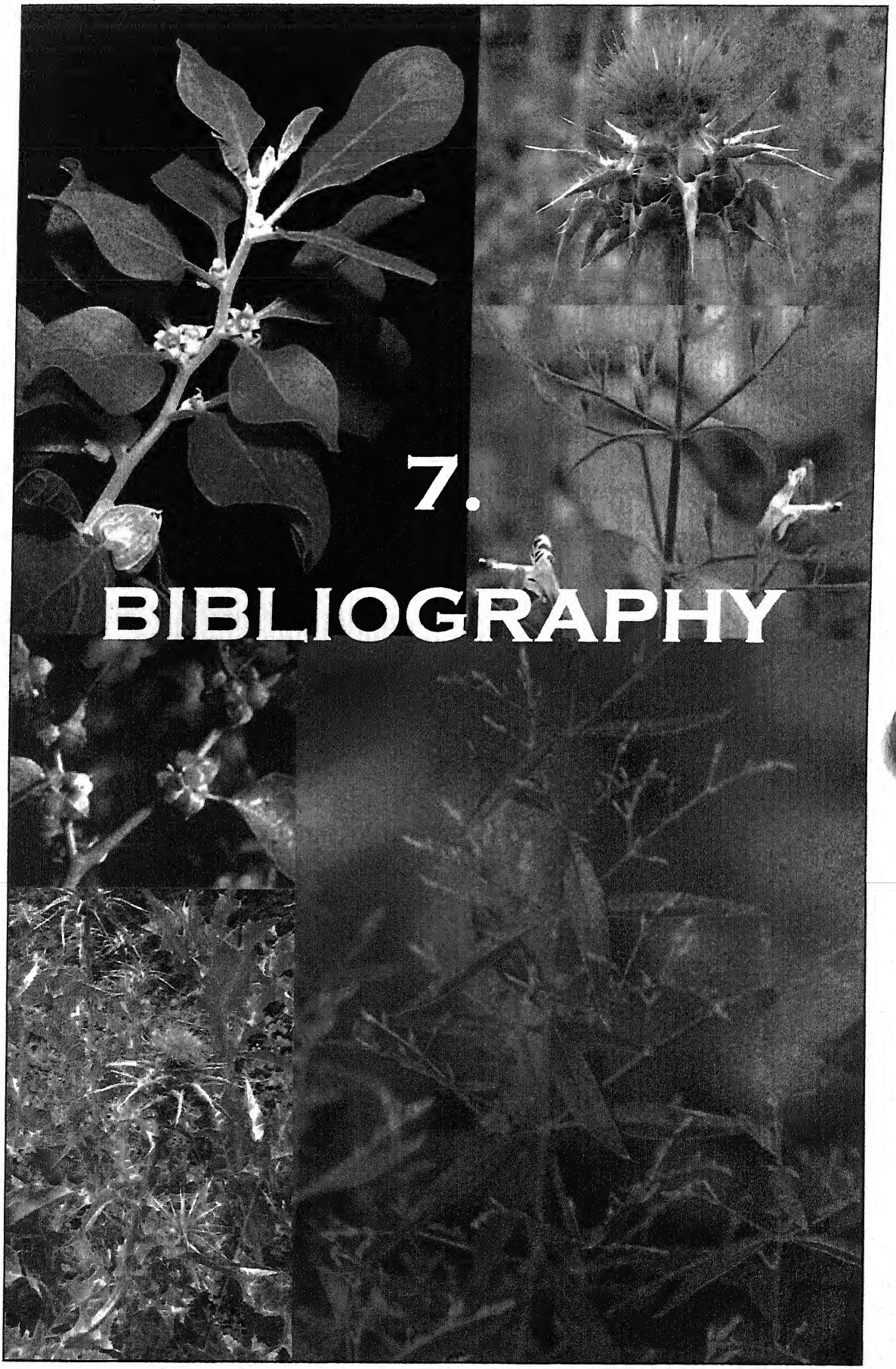
The proteomic investigation of *Silybum marianum* β-glucosidase was performed on MALDI-TOF and the peptide fragment amino acid sequence analysis revealed it to contain at least two sequence motifs VTPSNEVH and KRSEESNF that very strongly matched with β-glucosidases. The multiple sequence alignment revealed that these two sequence motifs shared highly conserved match with *Stigmatella aurantiaca* 1,4-β-D-glucan glucohydrolase D, *Bacillus thuringiensis* 6-aminohexanoate-dimer hydrolase, *Nitrosococcus oceani* ATCC 19707 Glycoside hydrolase, family 57, and *Bacillus halodurans* β-glucosidase. Amino acids T^{767}, P^{768}, N^{771}, E^{772} from *Stigmatella aurantiaca* and T^{396}, P^{397}, N^{398}, and E^{399} from *Bacillus* thuringiensis; and S^{246}, E^{248}, and S^{249} from *Nitrosococcus oceani* and S^{386}, E^{388}, and S^{389} from *Bacillus halodurans* were matching completely (highly conserved) from two respective fragments of *Silybum marianum* β-glucosidase.

The *Silybum marianum* β-glucosidase purification preparation contained a co-purified 74.1 kDa polypeptide which was revealed to be a putative fucosyltransferase. The peptide fragment analysis revealed to contain at least two sequence motifs YYEAYLSHADEK and TTPDPSCGR which ere highly conservatively matching with fucosyltransferases of *Arabidopsis thaliana* (xyloglucan fucosyltransferase and putative fucosyltransferase 10), *Oryza sativa* (putative xyloglucan fucosyltransferase and galactoside 2-α-L-fucosyltransferase), *Populus tremula* x *Populus alba* (α-1,2-fucosyltransferase).

Some of the kinetic or catalytic properties for the purified *Withania somnifera, Andrographis paniculata* and *Silybum marianum* enzymes were noted to be novel when comprehensively compared with its counterparts from plant, animal and microbial counterparts. These included high substrate affinity and catalytic efficiency, lack of glucose inhibition, substrate specificity, ferric ion activation, diminuted sensitivity to gluconolactone inhibition, specific transglycosylation etc.

Significance of building-up of a library of catalytically and/or kinetically novel and diverse plant β-glucosidases for structural investigation to understand naturally evolved mechanics of catalysis has been indicated. This study provided insighting biochemical/catalytic attributes for β-glucosidase from selected medicinal plants for exploration into the realm of metabolism and structural *versus* functional features.

In summation, such functional catalytic measures of enzyme can be applied to explore the tremendous potential of the plant β-glucosidase to develop a library of catalytically characteristic members of the family out of their huge repertoire expected to exist in plants for not only structure functional understanding but also for biotransformation applications. The further molecular characterizations data on the enzyme are very helpful in elaborating the catalytic aspects of the proteomic structure of enzyme including the molecular bases of diversity in substrate specificity. The generated information will advance and widen the potential metabolic applications of plant β-glucosidase. The correlations will embellish the enzyme functionality *in planta.*



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BIBLIOGRAPHY

Akiyama, T., Kaku, H. and Shibuya, N. (1998). A cell wall-bound β-glucosidase from germinated Rice: purification and properties. *Phytochemistry* 48: 49-54.

Amouri, B. and Gargouri, A. (2006). Characterization of a novel β-glucosidase from a *Stachybotrys* strain. *Biochem. Engg. J.* 32: 191–197.

Anbalagan, K. and Sadique, J. (1981). Influence of an Indian medicine (Ashwagandha) on acutephase reactants in inflammation. *Ind. J. Exp. Biol.* 19: 245-249.

Babcock, G.W. and Esen, A. (1994). Substrate specificity of maize β-glucosidase. *Plant Sci.* 101: 31-39.

Barbagallo, R.N., Spagna, G., Palmeri, R. and Torriani, S. (2004). Assessment of β-glucosidase activity in selected wild strains of *Oenococcus oeni* for malolactic fermentation. *Enz. Microb. Technol.* 34: 292–296.

Barbagallo, R.N., Palmeri, R., Fabiano, S., Rapisarda, P. and Spagna, G. (2007). Characteristic of β-glucosidase from Sicilian blood oranges in relation to anthocyanin degradation. *Enz. Microb. Technol.* 41: 570–575.

Bauer, M.W. and Kelly, R.M. (1998). The family 1 β-glucosidases from *Pyrococcus furiosus* and *Agrobacterium faecalis* share a common catalytic mechanism. *Biochemistry* 37: 17170-17178.

Bennett, R.N., Kiddle, G. and Wallsgrove, R.M. (1997). Biosynthesis of benzylglucosinolate, cyanogenic glucosides and phenylpropanoids in *Carica papaya*. *Phytochemistry* 45: 59–66.

Berrin, J.G., McLauchlan, W.R., Needs, P., Williamson, G., Puigserver, A., Kroon, P.A. and Juge, N. (2002). Functional expression of human liver cytosolic β-glucosidase in *Pichia pastoris*: Insights into its role in the metabolism of dietary glucosides. *Eur. J. Biochem.* 269: 249-258.

Beutler, E. (1992). Gaucher disease: new molecular approaches to diagnosis and treatment. *Science* 256: 794-798.

Bhatia, Y., Mishra, S. and Bisaria, V.S. (2002). Microbial β-Glucosidases: Cloning, Properties, and Applications. *Crit. Rev. Biotech.* 22: 375–407.

Bhattacharya, S.K., Satyan, K.S. and Ghosal, S. (1997). Antioxidant activity of glycowithanolides from *Withania somnifera*. *Indian J. Exp. Biol.* 35: 236–239.

Bhattacharya, S.K., Goel, R.K., Kaur, R. and Ghosal, S. (1987). Antistress activity of sitoindosides VII and VIII, new acylsterylglucosides from *Withania somnifera*. *Phytotherapy Res.* 1: 32-39.

Blum, H., Beier, H. and Gross, H.J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8: 93-99.

Brandt, V., Geerlings, A., Tits, M., Delaude, C., van der Heijden, R., Verpoorte, R. and Angenot, L. (2000). New strictosidine beta-glucosidase from *Strychnos mellodora*. *Plant Physiol. Biochem.* 38: 187-192.

Brzobohaty, B., Moore, I., Kristoffersen, P., Bako, L., Campos, N., Schell, J. and Palme, K. (1993). Release of active cytokinin by a β-glucosidase localized to the maize root meristem. *Science* 262: 1051-1054.

Calabrese, C., Berman, S.H., Babish, J.G., Ma, X., Shinto, L., Dorr, M., Wells, K., Wenner, C.A. and Standish, L.J. (2000). A phase I trial of andrographolide in HIV positive patients and normal volunteers. *Phytotherapy Res.* 14: 333–338.

Cameron, R.G., Manthey, J.A., Baker, R.A. and Grohmann, K. (2001). Purification and characterization of a β-Glucosidase from *Citrus sinensis* var. Valencia fruit tissue. *J. Agric. Food Chem.* 49: 4457 -4462

Chatterjee, A. and Pakrashi, S.C. (1995). The Treatise on Indian Medicinal Plants. 4: 208-212.

Chuankhayan, P., Hua, Y., Svasti, J., sakdarat, S., Sullivan, P.A. and Cairns, J.R.K. (2005). Purification of an isoflavonoid 7-*O*-β-apiosyl-glucoside β-glycosidase and its substrates from *Dalbergia nigrescens* Kurtz. *Phytochemistry* 66: 1880-1889.

Clarke, A.J., Bray, M.R. and Strating, H. (1993). β-glucosidases, β-glucanases, and xylanases. In A. Esen Ed. β-Glucosidases: Biochemistry and Molecular Biology, ACS Symposium Series 533. American Chemical Society, Washington D.C. pp. 27-41.

Conn, E.E. (1981). Cyanogenesis glycosides. In: Stumpf, P.K., Conn, (Eds) Biochemistry of Plants, Academic Press, New York 7: 479-500.

Costa, G., Decou, R. and Lhernould, S. (2007). The multigenic family of fucyltransferase from poplar tree. Submitted (JUL-2007) to the EMBL/GenBank/DDBJ databases.

Costache, M., Apoil, P., Cailleau, A., Elmgreni, A., Larsoni, G., Henry, S., Blancher, A., Iordachescu, D., Oriol, R. and Mollicone, R. (1997). Evolution of fucosyltransferase genes in vertebrates. *J. Biol. Chem.* 272: 29721–29728.

Cote, F. and Hahn, M.G. (1994). Oligosaccharins: structures and signal transduction. *Plant Mol. Biol.* 26: 1379–1411.

Cuevas, L., Niemeyer, H.M. and Jonsson, L.M.V. (1992). Partial purification and characterization of a hydroxamic acid glucoside β-glucosidase from maize. *Phytochemistry* 31: 2609-2612.

Davis, B.G. (2000). Recent developments in oligosaccharide synthesis. *J. Chem. Soc. Perkin Trans. 1* 2137-2160.

Day, A.G. and Withers, S.G. (1986). The purification and characterization of a β-glucosidase from *Alcaligenes faecalis*. *Can. J. Biochem. Cell Biol.* 64: 914-922.

Depre, D., Dueffels, A., Green, L.G., Lenz, R., Ley, S.V. and Wong, C.H. (1999). Synthesis of glycans from the glycodelins: two undeca-, two deca-, three nona-, an octa- and a heptasaccharide. *Chem. Eur. J.* 5: 3326-3340.

Devi, P.U., Sharada, A.C. and Solomon, F.E. (1995). *In vivo* growth inhibitory and radiosensitizing effects of withaferin A on mouse Ehrlich ascites carcinoma. *Cancer Lett.* 95: 189-193.

Dharmawardhana, D.P., Ellis, B.E. and Carlson, J.E. (1995). A β-glucosidase from lodgpole pine xylem specific for the lignin precursor coniferin. *Plant Physiol.* 107: 331-339.

Dignum, M.J.W., Heijden, R., Kerler, J., Winkel, C. and Verpoorte, R. (2004). Identification of glucosides in green beans of *Vanilla planifolia* Andrews and kinetics of vanilla β-glucosidase. *Food Chem.* 85: 199–205.

Doucet, J.P. and Trifaro, J.M. (1988). A discontinuous and highly porous sodium dodecyl sulfate-polyacrylamide slab gel system of high resolution. *Anal. Biochem.* 168: 265-271.

Esen, A. (1992). Purification and characterization of Maize (*Zea mays* L.) β-glucosidase. *Plant Physiol.* 98: 174-182.

Esen, A. and Stetler, D.A. (1993). Subcellular localization of maize β-glucosidase. *Maize Genet. Coop. News Lett.* 67: 19-20.

Estibalitz, O., Richard, O.A. and Ioannis, Z. (2001). The role of β-glucosidase in biosynthesis of 2,5-dimethyl-4-hydroxy-3 (2H)-furanone in strawberry. *Flav. Frag. J.* 16: 81-84.

Falk, A. and Rask, L. (1999). Expression of a Zeatin-*O*-Glucoside-degrading β-glucosidase in *Brassica napus*. *Plant Physiol.* 108: 1369-1377.

Fenwick, G.R., Heaney, R.K. and Mullin, W.J. (1983). Glucosinolates and their breakdown products in food and food plants. *CRC Crit. Rev. Food Sci. Nutr.* 18: 123-201.

Fieldes, M.A. and Gerhardt, K.E. (1994). An examination of the β-glucosidase (linamerase) banding pattern in flax seedling using Ferguson plots and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Electrophoresis* 15: 654-661.

Fischer, L. and Peissker, F. (1998). A covalent two-step immobilization technique using itaconic anhydride. *Applied Microb. Biotech.* 49: 129–135.

Flora, K., Hahn, M., Rosen, H. and Benner, K. (1998). Milk thistle (*Silybum marinum*) for the theraply of liver disease. *Am. J. Gastro.* 93: 139-143.

Fort, S., Boyer, V., Greffe, L., Davies, G.J., Moroz, O., Christiansen, L., Schuelein, M., Cottaz S. and Driguez, H. (2000). Highly efficient synthesis of β(1-4)-oligo- and -polysaccharides using a mutant cellulase. *J. Am. Chem. Soc.* 122: 5429-5437.

Fowler, T. (1993). Deletion of the *Tricoderma reesei* β-glucosidase gene *bgl1*. In A. Esen ed. β-Glucosidases: Biochemistry and Molecular Biology. ACS symposium series 533. American Chemical Society, Washington D.C. pp. 56-65.

Fraschini, F., Demartini, G. and Esposti, D. (2002). Pharmacology of Silymarin. *Clin. Drug Invest.* 22: 51-65.

Gazak, R., Svobodova, A., Psotova, J., Sedmera, P., Prikriylova, V., Walterova, D. and Kren, V. (2004). Oxidised derivatives of silybin and their antiradical and antioxidant activity. *Bioorg. Med. Chem.* 12: 5677-5687.

Gerardi, C., Blando, F., Santino, A. and Zacheo, G. (2001). Purification and characterization of a β-glucosidase abundantly expressed in ripe sweet cherry (*Prunus avium* L.) fruit. *Plant Sci.* 160: 795-805.

Gerasimenko, I., Sheludko, Y., Ma, X. and Stockigt, J. (2002). Heterologous expression of a *Rauvolfia* cDNA encoding strictosidine glucosidase, a biosynthetic key to over 2000 monoterpenoid indole alkaloids. *Eur. J. Biochem.* 269: 2204-2213.

Gueguen, Y., Chemardin, P., Janbon, G., Arnaud, A. and Galzy, P. (1996). A very efficient β-glucosidase-catalyst for the hydrolysis of flavor precursors process of wines and fruit juices. *J. Agric. Food Chem.* 44: 2336–2340.

Gunata, Y.Z., Bayonove, C.L., Baumes, R. and Cordonnier, R. (1985). The aroma of grapes. Extraction and determination of free and glycosidically bound fractions of some grape aroma components. *J. Chromatogr.* 331: 83-90.

Gupta, G.L. and Rana, A.C. (2007). *Withania somnifera* (Ashwagandha): A Review. *Pharmacog. Rev.* 1: 129-136.

Gus-Mayer, S., Brunner, H., Schneider-Poetsch, H.A. and Rudiger, W. (1996). Avenacosidase from oat: purification, sequence analysis and biochemical characterization of a new member of the BGA family of β-glucosidases. *Plant Mol. Biol.* 26: 909–921.

Han, C.S., Xie, G., Challacombe, J.F., Altherr, M.R., Bhotika, S.S., Bruce, D., Campbell, C.S., Campbell, M.L., Chen, J., Chertkov, O., Cleland, C., Dimitrijevic, M., Doggett, N.A., Fawcett, J.J., Glavina, T., Goodwin, L.A., Hill, K.K., Hitchcock, P., Jackson, P.J., Keim, P., Kewalramani, A.R., Longmire, J., Lucas, S., Malfatti, S., McMurry, K., Meincke, L.J., Misra, M., Moseman, B.L., Mundt, M., Munk, A.C., Okinaka, R.T., Parson-Quintana, B., Reilly, L.P., Richardson, P., Robinson, D.L., Rubin, E., Saunders, E., Tapia, R., Tesmer, J.G., Thayer, N., Thompson, L.S., Tice, H., Ticknor, L.O., Wills, P.L., Brettin, T.S. and Gilna, P. (2006). Pathogenomic sequence analysis of Bacillus cereus and Bacillus thuringiensis isolates closely related to *Bacillus anthracis*. *J. Bacteriol.* 188: 3382-3390.

Hansson, T., Andersson, M., Wehtje, E. and Adlercreutz, P. (2001). Influence of water activity on the competition between β-glycosidasecatalysed transglycosylation and hydrolysis in aqueous hexanol. *Enz. Microb. Technol.* 29: 527–534

Hays, W.S., VanderJagt, D.J., Bose, B., Serianni, A.S. and Glew, R.H. (1998). Catalytic mechanism and specificity for hydrolysis and transglycosylation reactions of cytosolic β-glucosidase from guinea pig liver. *J. Biol. Chem.* 273: 34941-34948.

Hemscheidt, T. and Zenk, M.H. (1980). Glucosidases involved in indole alkaloid biosynthesis of Catharanthus cell cultures. *FEBS Lett.* 110: 187-191.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid similarities. *Biochem. J.* 280: 309-316.

Henrissat, B. (1996). Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* 316: 695-696.

Henrissat, B. and Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino-acid-sequence similarities *Biochem. J.* 293: 781-788.

Henrissat, B. and Romeus, A. (1995). Families, superfamilies and subfamilies of glycosyl-hydrolases. *Biochem. J.* 311: 350-351.

Holt, Stephen, M.D. and Comac, L. (1998). Miracle herbs: how herbs combine with modern medicine to treat cancer, heart disease, AIDS, and more. Caro Publishing Group.

Hosel, W., Tober, I., Eklund, S.H. and Conn, E.E. (1987). Characterization of β-glucosidases with high specificity for the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L) Moench seedlings. *Arch Biochem. Biophys.* 252: 152-162.

Hrmova, M., Gori, R.D., Smith, B.J., Fairweather, J.K., Driguez, H., Varghese, J.N. and Fincher, G.B. (2002). Structural basis for broad substrate specificity in higher plant β-D-glucan glucohydrolases. *The Plant Cell* 14: 1033–1052.

Hrmova, M., Varghese, J.N., Hoj, P.B. and Fincher, G.B. (1998). Crystallization and preliminary X-ray analysis of β-D-glucan exohydrolase isoenzyme ExoI from barley (*Hordeum vulgare*). *Acta Crystallogr. Sect. D* 54: 687–689.

Hsieh, M.C. and Graham, T.L. (2001). Partial purification and characterization of a soybean β-glucosidase with high specific activity towards isoflavone conjugates. *Phytochemistry* 58: 995-1005.

Hu, Y., Luan, H., Hao, D., Xiao, H., Yang, S. and Yang, L. (2007). Purification and characterization of novel ginsenoside-hydrolyzing β-D-glucosidase from the China white jade snail (*Achatina fulica*). *Enz. Microb. Technol.* 40, 1358-1360.

Hughes, M.A. (1993). Molecular genetics of plant cyanogenic β-glucosidases. In A. Esen Ed. β-Glucosidases: Biochemistry and Molecular Biology, ACS symposium series 533. American Chemical Society, Washington D.C. pp. 153-169.

Isorna, P., Polaina, J., Garcia, L.L., Canada, F.J., Gonzalez, B. and Aparicio, J.S. (2007). Crystal structures of *Paenibacillus polymyxa* β-glucosidase B complex reveals the molecular basis of substrate specificity and give new insights into the catalytic machinery of family I glycosidases. *J. Mol. Biol.* 371: 1204-1218.

Jones, D.A. (1988). Cyanogenesis in animal-plant interactions. In: D. Evered and S. Harnett (Editors), Cyanide Compounds in Biology. Ciba Foundation Symposia, New York 140: 151-170.

Kakes, P. (1985). Linamarase and other β-glucosidases are present in the cell walls of *Trifolium repens* L. leaves. *Planta* 166: 156-160.

Kakes, P. (1993). Function and variation of the β-glucosidase Linamarase in natural populations of *Trifolium repens*. β-Glucosidases: Biochemistry and Molecular Biology. In A. Esen ed. ACS Symposium Series 533. American Chemical Society, Washington D.C. pp.145-152.

Kannan, T., Loganathan, D., Bhatia, Y., Mishra, S. and Bisaria, V.S. (2004). Transglycosylation catalyzed by almond β-glucosidase and cloned *Pichia etchellsii* β-glucosidase II using glycosylasparagine mimetics as novel acceptors. *Biocat. Biotrans.* 22: 1-7.

Kapil, A., Koul, I.B., Banerjee, S.K. and Gupta, B.D. (1993). Antihepatotoxic effects of major diterpenoid constituents of *Andrographis paniculata. Biochem. Pharma.* 46: 182-85.

Karnchanatat, A., Petsom, A., Sangvanich, P., Piaphukiew, J., Whalley, A.J.S., Reynolds, C.D. and Sihanonth, P. (2007). Purification and biochemical characterization of an extracellular β-glucosidase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.)Rehm. *FEMS Microbiol. Lett.* 270: 162–170.

Kelly, R.J., Rouquier, S., Giorgi, D., Lennon, G.G. and Lowe, J.B. (1995). Sequence and expression of a candidate for the human secretor blood group alpha(1,2)fucosyltransferase gene (FUT2). Homozygosity for an enzyme-inactivating nonsense mutation commonly correlates with the non-secretor phenotype. *J. Biol. Chem.* 270: 4640-4649.

Kempton, J.B. and Withers, S.G. (1992). Mechanism of *Agrobacterium* β-glucosidase: kinetic studies. *Biochemistry* 31: 9961-9969.

Keresztessy, Z., Brown, K., Dunn, M.A. and Hughes, M.A. (2001). Identification of essential active-site residues in the cyanogenic β-glucosidase (linamarase) from cassava (*Manihot esculenta* Crantz) by site-directed mutagenesis. *Biochem. J.* 353: 199-205.

Kim, S.Y., Kim, Y.W., Hegerl, R., Cyrkla, M. and Kim, I.S. (2005). Novel type of enzyme multimerization enhances substrate affinity of oat β-glucosidase. *J. Structural Biol.* 150: 1–10.

Klotz, M.G., Arp, D.J., Chain, P.S.G., El-Sheikh, A.F., Hauser, L.J., Hommes, N.G., Larimer, F.W., Malfatti, S.A., Norton, J.M., Poret-Peterson, A.T., Vergez, L.M. and Ward, B.B. (2006). Complete genome sequence of the marine, chemolithoautotrophic, ammonia-oxidizing bacterium *Nitrosococcus oceani* ATCC 19707. *Appl. Environ. Microbiol.* 72: 6299-6315.

Koeller, K. and Wong, C.H. (2000). Chemoenzymatic synthesis of sialyl-trimer-Lewis X. *Chem. Eur J.* 6: 1243-1251.

Konno, H., Yamasaki, Y. and Katoh, K. (1996). A β-glucosidase associated with cell walls from cell suspension cultures of carrot. *Phytochemistry* 43: 1157-1161.

Koshland, D.E.J. (1953). Steriochemistry and the mechanism of enzymatic reactions. *Biol. Rev.* 28: 416-436.

Kosina, P., Kren, V., Gebhardt, R., Grambal, F., Ulrichova, J. and Walterova, D. (2002). Antioxidant Properties of Silybin Glycosides. *Phytother. Res.* 16, S33–S39.

Kren, V. and Martankova, L. (2001). Glycosides in Medicine: "The Role of Glycosidic Residue in Biological Activity". *Curr. Med. Chem.* 8: 1313-1338.

Kren, V. and Walterova, D. (2005). Silybin and silymarin – new effects and applications. *Biomed. Papers* 149: 29–41.

Kren, V., Minghetti, A., Sedmera, P., Havlicek, V., Prikrylova, V. and Crespi-Perellino N. (1998). Glucosylation of silybin by plant cell cultures of *Papaver somniferum* var. setigerum. *Phytochemistry* 47: 217–220.

Kukowska-Latallo, J.F., Larsen, R.D., Nair, R.P. and Lowe, J.B. (1990). A cloned human cDNA determines expression of a mouse stage-specific embryonic antigen and the Lewis blood group alpha(1,3/1,4)fucosyltransferase. *Genes Dev.* 4: 1288-1303.

Kuro, O.M., Matsumura, Y., Aizawa, H., Kawaguchi, H., Suga, T., Utsugi, T., Ohyama, Y., Kurabayashi, M., Kaname, T. and Kume, E. (1997). Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 390: 45-51.

Langston, J., Sheehy, N. and Xu, F. (2006). Substrate specificity of *Aspergillus oryzae* family 3 β-glucosidase. *Biochim. Biophys. Acta* 1764: 972–978.

Lau, A.T.Y. and Wong, W.K.R. (2001). Purification and characterization of a major secretory cellobiase, Cba2, from *Cellulomonas biazotea*. *Prot. Express. Purif.* 23: 159-166.

Lawson, S.L., Wakarchuk, W.W. and Withers, S.G. (1996). Effects of both shortening and lengthening the active site nucleophile of *Bacillus circulans* xylanase on catalytic activity. *Biochemistry* 35: 10110-10118.

Leah, R., Kigel, J., Svendsen, I. and Mundy, J. (1995). Biochemical and molecular characterization of barley seed β-glucosidase. *J. Biol. Chem.* 270: 15789-15797.

Lecas, M., Gunata, Z., Sapis, J.C. and Bayonove, C. (1991). Purification and partial characterization of β-glucosidase from grapes. *Phytochemistry* 30: 451–454.

Legler, G., Sinnott, M.L. and Withers, S.G. (1980). Catalysis by β-glucosidase A3 of *Aspergillus wentii*. *J. Chem. Soc. Perkin Trans.* 11: 1376-1383.

Leslie, E.M., Mao, Q., Oleschuk, C.J., Deeley, R.G. and Cole, S.P.C. (2001). Modulation of multidrug resistance protein 1 (MRP1/ABCC1) transport and ATPase activities by interaction with dietary flavonoids. *Mol. Pharmacol.* 59: 1171–1180.

Levy, S., Maclachlan, G. and Staehelin, L. A. (1997). Xyloglucan sidechains modulate binding to cellulose during *in vitro* binding assays as predicted by conformational dynamics simulations. *Plant J.* 11: 373–386.

Levy, S., York, W.S., Stuike-Prill, R., Meyer, B. and Staehelin, L.A. (1991). Simulations of the static and dynamic molecular conformations of xyloglucan. The role of the fucosylated sidecham in surface specific sidechain folding. *Plant J.* 1: 195–215.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with Folin Phenol reagent. *J. Biol. Chem.* 193: 265-275.

Luijendijk, T.J.C., Stevens, L.H. and Verpoorte, R. (1998). Purification and characterization of strictosidine β-D-glucosidase from *Catharanthus roseus* cell suspension cultures. *Plant Physiol. Biochem.* 36: 419-425.

Mallikarjuna, G., Dhanalakshmi, S., Singh, R.P., Agarwal, C. and Agarwal, R. (2004). Silibinin protects against photocarcinogenesis *via* modulation of cell cycle regulators, mitogen-activated protein kinases, and Akt signaling. *Cancer Res.* 64: 6349–6356.

Martin, S.F. (2003). Unified strategy for the synthesis of C-aryl glycosides. *Pure Appl. Chem.* 75: 63–70.

Matsuda, H., Murakami, T., Kishi, A. and Yoshikawa, M. (2001). Structures of Withanosides I, II, III, IV, V, VI, and VII, new withanolide glycosides, from the

roots of Indian *Withania somnifera* DUNAL. and inhibitory activity for tachyphylaxis to clonidine in isolated Guinea-Pig ileum. *Bioorg. Med. Chem.* 9: 1499–1507.

Matsuda, T., Kuroyanagi, M., Sugiyama, S., Umehara, K., Ueno, A. and Nishi, K. (1994). Cell differentiation-inducing diterpenes from *Andrographis paniculata* Nees. *Chem. Pharm. Bull.* (Tokyo) 42: 1216-1225.

Matsumura, S., Nakamura, T., Yao, E. and Toshima, K. (1999). Biocatalytic one-step synthesis of *n*-octyl β-D-xylotrioside and xylobioside from xylan and *n*-octanol in supercritical carbon dioxide. *Chem. Letts.* 581-582.

May, U. and Kreis, W. (1997). Purification and characterization of the cardenolide-specific β-glycohydrolase CGH I from *Digitalis lanata* leaves. *Plant Physiol. Biochem.* 35: 523–532.

McMahon, J.M., White, W.L.B. and Sayre, R.T. (1995). Cyanogenesis in cassava (*Manihot esculenta* Crantz). *J. Exp. Bot.* 46: 731-741.

McMahon, L.G., Nakano, H., Levy, M.D. and Gregory III, J.F. (1997). Cytosolic pyridoxine-β-D-glucoside hydrolase from porcine jejunal mucosa. Purification, properties, and comparison with broad specificity β-glucosidase. *J. Biol. Chem.* 272: 32025-32033.

Minami, Y., Shigeta, Y., Tokumoto, U., Tanaka, Y., Sakakibara, K.Y., Oh-oka, H. and Matsubara, H. (1999). Cloning, sequencing, characterization, and expression of a β-glucosidase cDNA from the indigo plant. *Plant Sci.* 142: 219–226.

Misra, P., Pal, N.L., Guru, P.Y., Katiyar, J.C., Srivastava, V. and Tandon, J.S. (1992). Antimalarial activity of *Andrographis paniculata* (Kalmegh) against *Plasmodium berghei* NK 65 in *Mastomys natatensis*. *Int. J. Pharmacogenomics* 30: 263–274.

Mkpong, O.E., Yan, H., Chism, G. and Sayre, R.T. (1990). Purification, characterization and localization of linamarase in cassava. *Plant physiol.* 93: 176-181.

Murray, P., Aro, N., Collins, C., Grassick, A., Penttilä, M., Saloheimo. M. and Tuohy, M. (2004). Expression in *Trichoderma reesei* and characterisation of a thermostable family 3 β-glucosidase from the moderately thermophilic fungus *Talaromyces emersonii*. *Prot. Express. Purif.* 38: 248-257.

Nakkharat, P. and Haltrich, D. (2006). Purification and characterization of an intracellular enzyme with β-glucosidase and β-galactosidase activity from the thermophilic fungus *Talaromyces thermophilus* CBS 236.58. *J. Biotech.* 123: 304-313.

Natsuka, S., Gersten, K.M., Zenita, K., Kannagi, R. and Lowe, J.B. (1994). Molecular cloning of a cDNA encoding a novel human leukocyte α-1,3-fucosyltransferase capable of synthesizing the sialyl Lewis x determinant. *J. Biol. Chem.* 269: 16789-16794.

Niemeyer, H.M. (1988). Hydroxamic acid (4-hydroxy-1,4-benzoxazin-3-ones), defence chemicals in the gramineae. *Phytochemistry* 27: 3349-3358.

Niemeyer, H.M., Calcaterra, N.B. and Roveri, O.A. (1986). Inhibition of mitochondria energy-linked reactions by 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid from Gramineae. *Biochem. Pharmacol.* 15: 3909-3914.

Nierman W.C. (2006). Nucleotide sequence, *Stigmatella aurantiaca* DW4/3-1 1,4-β-D-glucan glucohydrolase (Fragment). Submitted (APR-2006) to the EMBL/GenBank/DDBJ databases.

Ohashi, T., Boggs, S., Robbins, P., Bahnson, A., Patrene, K., Wei, F., Wei, J., Li, J., Lucht, L., Fei, Y., Clark, S., Kimak, M., He, H., Mowery-Rushton, P. and Barranger, J.A. (1992). Efficient transfer and sustained high expression of the human glucocerobrosidase gene in mice and their functional macrophages following transplantation of the bone marrow transduced by a retroviral vector. *Proc. Natl. Acad. Sci.* USA 89: 11332-11336.

Opassiri, R., Cairns, J.R.K., Akiyama, T., Aswapati, O.W., Svasti, J. and Esen, A. (2003). Characterization of a rice β-glucosidase highly expressed in flower and germinating shoot. *Plant Sci.* 165: 627-638.

Opassiri, R., Hua, Y., Aswapati, O.W., Akiyama, T., Svasti, J., Esen, A. and Cairns, J.R.K. (2004). β-Glucosidase, exo-β-glucanase and pyridoxine transglucosylase activities of rice BGlu1. *Biochem. J.* 379: 125–131.

Oxtoby, E., Dunn, M.A., Pancoro. A. and Hughes, M.A. (1991). Nucleotide and derived amino acid sequence of the cyanogenic β-glucosidase (Linamarase) from white clover (*Trifolium repens* L.). *Plant Mol. Bio.* 17: 209-219.

Pardo, A.G. and Forchiassin, F. (1999). Factors influencing β-glucosidase production, activity and stability in *Nectria catalinensis*. *Folia Microbiolia* 44: 71–76.

Perrin, R.M., DeRocher, A.E., Bar-Peled, M., Zeng, W., Norambuena, L., Orellana, A., Raikhel, N.V. and Keegstra, K. (1999). Xyloglucan fucosyltransferase, an enzyme involved in plant cell wall biosynthesis. *Science* 284: 1976–1979.

Pontoh, J. and Low, N.H. (2002). Purification and characterization of β-glucosidase from honey bees (*Apis mellifera*). *Insect Biochem. Mol. Biol.* 32: 679–690.

Poulton, J.E. (1988). Localization and catabolism of cyanogenic glycosides. In: D. Evered and S. Harnett (Editors), Cyanide Compounds in Biology. Ciba Foundation Symposia, NewYork 140: 67-81.

Poulton, J.E. (1989). Toxic compounds in plant foodstuffs: cyanogens. In J.E. Kinsella, W.G. Soucie, eds, Food Proteins. The American Oil Chemist' Society, Champaign, I.L. pp. 381-401.

Poulton, J.E. (1990). Cyanogenesis in Plants. *Plant Physiol.* 94: 401-405.

Poulton, J.E. (1993). Enzymology of cyanogenesis in rosaceous stone fruits. In A. Esen ed, β-Glucosidases: Biochemistry and Molecular Biology, ACS symposium series 553, American Chemical Society, Washington D.C. pp. 170-190.

Prakash, J., Gupta, S.K. and Dinda, A.K. (2002). *Withania somnifera* root extract prevents DMBA-induced quamous cell carcinoma of skin in Swiss albino mice. *Nutr. Cancer* 42: 91-97.

Puri, A., Saxena, R., Saxena, R.P. and Saxena, K.C. (1993). Immunostimulant agents from *Andrographis paniculata. J. Nat. Prod.* 56: 995-999.

Reuveni, M., Zohar, S., Evnor, D. and Hetzroni, A. (1999). β-Glucosidase activity is involved in scent production in *Narcissus* flowers. *Plant Sci.* 147: 19–24.

Riou, C., Salmon, J.M., Vallier, M.J., Gunata, Z. and Barre, P. (1998). Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryzae. App. Env. Microbiol.* 64: 3607-3614.

Sallas, F. and Nishimura, S.I. (2000). Chemo-enzymatic synthesis of glycopolymers and sequential glycopeptides bearing lactosamine and sialyl Lewis x unit pendant chains. *J. Chem. Soc. Perkin Trans 1* 2091-2103.

Sarria, R.., Wagner, T.A., O'Neill, M.A., Faik, A., Wilkerson, C.G., Keegstra, K. and Raikhel, N.V. (2001). Characterization of a family of Arabidopsis genes related to xyloglucan fucosyltransferase1. *Plant Physiol.* 127: 1595-1606.

Sasaki, T., Matsumoto, T. and Katayose, Y. (2002). *Oryza sativa* nipponbare(GA3) genomic DNA, chromosome 9, BAC clone:B1045B05. Submitted (NOV-2002) to the EMBL/GenBank/DDBJ databases.

Schliebs, R., Liebmann, A. and Bhattacharya, S.K. (1997). Systemic administration of defined extracts from *Withania somnifera* (Indian Ginseng) and Shilajit differentially affects cholinergic but not glutamatergic and GABAergic markers in rat brain. *Neurochem. Int.* 30: 181-190.

Schreier, J.H. and Schreier, P. (1986). Purification and partial characterization of β-glucosidase from papaya fruit. *Phytochemistry* 10: 2271–2274.

Schubel, H., Stockigt, J., Feicht, R. and Simon, H. (1986). Partial purification and characterization of raucaffricine β-D-glucosidase from plant cell-suspension cultures of *Rauwolfia serpentina* BENTH. *Helv. Chim. Acta* 69: 538-547.

Seidle, H.F. and Huber, R.E. (2005). Transglucosidic reactions of the *Aspergillus niger* Family 3 β-glucosidase: Qualitative and quantitative analyses and evidence that the transglucosidic rate is independent of pH. *Arch Biochem. Biophys.* 436: 254-264.

Seigler, D.S., Maslin, B.R. and Conn, E.E. (1989). Cyanogenesis in the Leguminosae. Monographs in Systematic Botany from the Missouri Botanical Garden 29: 645-672.

Selmar, D., Lieberei, R., Biehl, B. and Voigh, J. (1987). *Hevea* linamarase, a nonspecific β-glycosidase. *Plant Physiol.* 83: 557-567.

Seto, N.O.L., Compston, C.A., Szpacenko, A. and Palcic, M.M. (2000). Enzymatic synthesis of blood group A and B trisaccharide analogues. *Carbohydrate Res.* 324: 161-169.

Sharma, A., Krishan, L. and Handa, S.S. (1992). Standardization of the Indian crude drug Kalmegh by high pressure liquid chromatographic determination of andrographolide. *Phytochem. Anal.* 3: 129-31.

Simos, G., Panagiotidis, C.A., Skoumbas, A., Choli, D., Ouzounis, C. and Georgatsos, J.G. (1994). Barley β-glucosidase: Expression during seed germination and maturation and partial amino acid sequences. *Biochim. Biophys. Acta* 1199: 52-58.

Singh, N., Nath, R. and Lata, A. (1982). *Withania somnifera* (ashwagandha), a rejuvenating herbal drug which enhances survival during stress (an adaptogen). *Int. J. Crude Drug Res.* 20: 29-35.

Singh, N., Singh, S.P. and Nath, R. (1986). Prevention of urethane-induced lung adenomas by *Withania somnifera* (L.) Dunal in albino mice. *Int. J. Crude Drug Res.* 24: 90-100.

Singh, P.K., Roy, S. and Dey. S. (2003). Antimicrobial activity of *Andrographis paniculata. Fitoterapia* 74: 692–694.

Sinnott, M.L. (1990). Catalytic mechanism of enzymic glycosyl transfer. *Chem. Rev.* 90: 1171-1202.

Siripong, P., Kongkathip, B., Preechanukool, K., Picha, P., Tunsuwan, K. and Taylor, W.C. (1992). Cytotoxic diterpenoid constituents from *Andrographis paniculata,* Nees leaves. *J. Sci. Soc.* Thailand 18: 187-194.

Somasundaram, S., Sadique, J. and Subramoniam, A. (1983). *In vitro* absorption of [^{14}C]leucine during inflammation and the effect of anti-inflammatory drugs in the jejunum of rats. *Biochem. Med.* 29: 259-264.

Srisomsap, C., Svasti, J., Surarit, R., Champattanachai, V., Sawangareetrakul, P., Boonpuan, K., Subhasitanont, P. and Chokchaichamnankit, D. (1996). Isolation and characterization of an enzyme with β-glucosidase and β-fucosidase activities from *Dalbergia cochinchinensis* Pierre. *J. Biochem.* 119: 585-590.

Stevens, L.H., Blom, T.J.M. and Verpoorte, R. (1993). Subcellular localization of tryptophan decarboxylase, strictosidin β-D-glucosidase in suspension cultured cells of *Catharanthus roseus* and *Tabernaemontana divaricata. Plant Cell Report* 573-576.

Sue, M., Ishihara, A. and Iwamura, H. (2000). Purification and characterization of a β-glucosidase from rye (*Secale cereale* L.) seedlings. *Plant Sci.* 155: 67-74.

Sue, M., Yamazaki, K., Yajima, S., Nomura, T., Matsukawa, T., Iwamura, H. and Miyamoto, T. (2006). Molecular and structural characterization of hexameric β-D-glucosidases in wheat and rye. *Plant Physiology*: 141, 1237–1247.

Svasti, J., Phongsak, T. and Sarnthima, R. (2003). Transglucosylation of tertiary alcohols using cassava β-glucosidase. *Biochem. Biophys. Res. Comm.* 305: 470–475.

Svasti, J., Srisomsap, C., Techasakul, S. and Surarit, R. (1999). Dalcochinin-8′-*O*-β-D-glucoside and its β-glucosidase enzyme from *Dalbergia cochinchinensis. Phytochemistry* 49: 628-632.

Takami, H., Nakasone, K., Takaki, Y., Maeno, G., Sasaki, R., Masui, N., Fuji, F., Hirama, C., Nakamura, Y., Ogasawara, N., Kuhara, S. and Horikoshi, K. (2000). Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. *Nucleic Acids Res.* 28: 4317-4331.

Tribolo, S., Berrin, J.G., Kroon, P.A., Czjzek, M. and Juge, N. (2007). The Crystal structure of human cytosolic β-glucosidase unravels the substrate aglycone specificity of a Family 1 glycoside hydrolase. *J. Mol. Biol.* 370: 964–975.

Trimbur, D.E., Warren, R.A.J. and Withers S.G. (1992). Region-directed mutagenesis of residues surrounding the active site nucleophile in β-glucosidase from *Agrobacterium faecalis*. *J. Biol. Chem.* 267: 10248-10252.

Varghese, J.N., Hrmova, M. and Fincher, G.B. (1999) Three-dimensional structure of a barley β-D-glucan exohydrolase, a family 3 glycosyl hydrolase. *Structure 7*: 79-190.

Vasserot, Y., Arnaud, A. and Galzy, P. (1995). Monoterpenyl glycosides in plant and their biotechnological transformation. *Acta Biotechnol.* 15: 77-95.

Venturi, L.L., Polizeli, M.D.L., Terenzi, H.F., Furriel, R.D.P.M. and Jorge, J.A. (2002). Extracellular β-D-glucosidase from *Chaetomium thermophilum* var. *coprophilum*: production, purification and some biochemical properties. *J. Basic Microbiol.* 2: 5–66.

Verdoucq, L., Czjzek, M., Moriniere, J., Bevan, D.R. and Esen, A. (2003). Mutational and structural analysis of aglycone specificity in Maize and Sorghum β-glucosidases. *J. Biol. Chem.* 278: 25055-25062.

Vetter, J. (2000). Plant cyanogenic glycosides. *Toxicon* 38: 11-36.

Villena, M.A., Iranzo, J.F.U. and Pe´rez, A.I.B.P. (2007). β-Glucosidase activity in wine yeasts: Application in enology. *Enz. Microb. Technol.* 40: 420–425.

Wang, G. and Withers, S.G. (1995). Substrate-assisted catalysis in β-glycosidases. *J. Am. Chem Soc.* 117: 10137-10138.

Warzecha, H., Obitz, P. and Stockigt, J. (1999). Purification, partial amino acid sequence and structure of the product of raucaffricine-*O*-β-D-glucosidase from plant cell cultures of *Rauwolfia serpentina*. *Phytochemistry* 49: 1099-1109.

Watanabe, T., Sato, T., Yoshioka, S., Koshijima, T. and Kuwahara, M. (1992) Purification and properties of *Aspergillus niger* β-glucosidase. *Eur. J. Biochem. 209*: 651-659.

Whitney, S.E.C., Brigham, J.E., Darke, A.H., Reid, J.S.G. and Gidley, M.J. (1995). In vitro assembly of cellulose/xyloglucan networks: ultrastructural and molecular aspects. *Plant J.* 8: 491–504.

Wiese, G. and Grambow, H. (1986). Indole-3-methanol-β-D-glucoside and Indole-3-carboxylic acid-β-D-glucoside are products of indole-3-acetic acid degradation in wheat leaf segments. *Phytochemistry* 25: 2451-2455.

Winterhalter, P. and Skouroumonis, G.K. (1997). Glycoconjucated aroma compounds: occurrence, role and biotechnological transformation. *Adv. Biochem. Eng. Biotechnol.* 55: 73-105.

Withers, S.G. (2001). Mechanisms of glycosyl transferases and hydrolases. *Carbohydrate Polymers* 44: 325–337.

Withers, S.G. and Street, I.P. (1988). Identification of a covalent α-D-glucopyranosyl enzyme intermediate formed on a β-glucosidase. *J. Am. Chem. Soc.* 110: 8551-8553.

Withers, S.G. and Street, I.P. (1989). β-Glucosidases: Mechanism and Inhibition. Chapter 43. Plant Cell Wall Polymers: *Bioener. Biodegr.* pp. 597-607.

Withers, S.G., Warren, R.A.J., Street, I.P., Rupitz, K., Kempton, J.B. and Aebersold, R. (1990). Unequivocal demonstration of the involvement of a glutamate residue as a nucleophile in the mechanism of a "retaining" glycosidase. *J. Am. Chem. Soc.* 112: 5887-5889.

Woodward, J. and Wiseman, A. (1982). Fungal and other β-D-glucosidases, their properties and applications. *Enz. Microb. Technol.* 4: 73-93.

Yasukochi, T., Fukase, K. and Kusumoto, S. (1999). 3-Nitro-2-pyridyl glycoside as donor for chemical glycosylation and its application to chemoenzymatic synthesis of oligosaccharide. *Tetrahedron Lett.* 40: 6591-6593.

Ye, X.S. and Wong, C.H. (2000). Anomeric reactivity-based one-pot oligosaccharide synthesis: a rapid route to oligosaccharide libraries. *J. Org. Chem.* 65: 2410-2431.

Yeoh, H.H. and Woo, H.C. (1992). Cassava leaf β-glucosidase specificity and inhibition. *Phytochemistry* 31: 2263-2265.

Yeoh, H.H. and Wee, Y.C. (1994). Some properties of β-glucosidases from tropical plant species. *Phytochemistry* 35: 1391-1393.

Yin, J. and Guo, L. (1993). Contemporary traditional Chinese medicine. Beijing: Xie Yuan.

Yu, H.Y., Xua, J.H., Lu, W.Y. and Lin, G.Q. (2007). Identification, purification and characterization of β-glucosidase from apple seed as a novel catalyst for synthesis of *O*-glucosides. *Enz. Microb. Technol.* 40: 354–361.

Yu, J., Wang, J., Lin, W., Li, S., Li, H., Zhou, J., Ni, P., Dong, W., Hu, S., Zeng, C., Zhang, J., Zhang, Y., Li, R., Xu, Z., Li, S., Li, X., Zheng, H., Cong, L., Lin, L., Yin, J., Geng, J., Li, G., Shi, J., Liu, J., Lv, H., Li, J., Wang, J., Deng, Y., Ran, L., Shi, X., Wang, X., Wu, Q., Li, C., Ren, X., Wang, J., Wang, X., Li, D., Liu, D., Zhang, X., Ji, Z., Zhao, W., Sun, Y., Zhang, Z., Bao, J., Han, Y., Dong, L., Ji, J., Chen, P., Wu, S., Liu, J., Xiao, Y., Bu, D., Tan, J., Yang, L., Ye, C., Zhang, J., Xu, J., Zhou, Y., Yu, Y., Zhang, B., Zhuang, S., Wei, H., Liu, B., Lei, M., Yu, H., Li, Y., Xu, H., Wei, S., He, X., Fang, L., Zhang, Z., Zhang, Y., Huang, X., Su, Z., Tong, W., Li, J., Tong, Z., Li, S., Ye, J., Wang, L., Fang, L., Lei, T., Chen, C., Chen, H., Xu, Z., Li, H., Huang, H., Zhang, F., Xu, H., Li, N., Zhao, C., Li, S., Dong, L., Huang, Y., Li, L., Xi, Y., Qi, Q., Li, W., Zhang, B., Hu, W., Zhang, Y., Tian, X., Jiao, Y., Liang, X., Jin, J., Gao, L., Zheng, W., Hao, B., Liu, S., Wang, W., Yuan, L., Cao, M., McDermott, J., Samudrala, R., Wang, J., Wong, GK. and Yang, H. (2005). The Genomes of *Oryza sativa*: a history of duplications. *PLoS Biol.* 3: 266-281.

Zanoelo, F.F., Lourdes M.D., Polizeli, T.D.M., Terenzi, H.F. and Jorge, J.A. (2004). β-Glucosidase activity from the thermophilic fungus *Scytalidium thermophilum* is stimulated by glucose and xylose. *FEMS Microbiol. Lett.* 240: 137–143.

Zárate, R., Bonavia, M., Geerlings, A., Heijden, R. and Verpoorte, R. (2001). Expression of strictosidine β-D-glucosidase cDNA from *Catharanthus roseus*, involved in the monoterpene indole alkaloid pathway, in a transgenic suspension culture of *Nicotiana tabacum*. *Plant Physiol. Biochem.* 39: 763–769.

Zeng, Y.C. and Elbein, A.D. (1998). Purification to homogeneity and properties of plant glucosidase I. *Arch Biochem. Biophys.* 355: 26-34.

Ziauddin, M., Phansalkar, N. and Patki, P. (1996). Studies on the immunomodulatory effects of ashwagandha. *J. Ethnopharmacol.* 50: 69-76.